## Remarks

### Rejection Under 35 U.S.C. § 112, first paragraph

Claims 7-12 and 15-25 were rejected under 35 U.S.C. § 112, first paragraph, as not being enabled. Applicants respectfully traverse this rejection to the extent that it is applied to the claims as amended.

***The Legal Standard***

The test of enablement is whether one of ordinary skill in the art could make and use the claimed invention from the disclosures in the patent coupled with information known in the art without undue experimentation. *United States v. Telectronics, Inc.*, 857 F.2d 778, 8 U.S.P.Q.2d 1217 (Fed. Cir. 1988); *In re Stephens*, 529 F.2d 1343, 199 U.S.P.Q. 659 (C.C.P.A. 1976). A patent need not teach, and preferably omits, what is well known in the art. *In re Buchner*, 929 F.2d 660, 661, 18 U.S.P.Q.2d 13321, 1332 (Fed. Cir. 1991); *Spectra-Physics, Inc. v. Coherent, Inc.*, 827 F.2d 1524, 3 U.S.P.Q.2d 1737 (Fed. Cir. 1987). The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation (*M.I.T. v. A.B. Fortia*, 774 F.2d 1104 (Fed. Cir. 1985)).

Whether undue experimentation is needed is not based upon a single factor; it is a conclusion reached by weighing many factors. These factors have been summarized in *In re Wands*, 858 F.2d 731, 8 U.S.P.Q.2d 1400 (Fed. Cir. 1988) and include, but are not limited to:

(1) The quantity of experimentation necessary (time and expense);

(2) The amount of direction or guidance presented;

(3) The presence or absence of working examples of the invention;

(4) The nature of the invention;

(5) The state of the prior art;

(6) The relative skill of those in the art;

(7) The predictability or unpredictability of the art; and

(8) The breadth of the claims.

The M.P.E.P. explains that "[i]t is improper to conclude that a disclosure is not enabling based on an analysis of only one of the above factors while ignoring one or more of the others." Thus, a conclusion of nonenablement must be based on the evidence as a whole, as related to each of these factors (see M.P.E.P. § 2164.01 (a)).

The fact that some experimentation is necessary does not preclude enablement; what is required is that the amount of experimentation "must not be unduly extensive." *Atlas Powder Co., v. E.I. DuPont De Nemours & Co.*, 750 F.2d 1569, 1576, 224 USPQ 409, 413 (Fed. Cir.1984). There is no requirement for examples.

***Claims 7-12 and 15-25 are enabled***

Claims 7-12 and 15-25 were rejected under 35 U.S.C. § 112, first paragraph for not being enabled for (1) *in vivo* methods for targeted recombination in which the triple-helix-forming oligonucleotide (TFO) has a Kd of more than $2 \times 10^{-7}$ (2) targeted recombination *in vivo* or methods in which targeted recombination produces heritable changes in the genome of an intact animal or human.

(1) Claims 7 and 15 have been amended as the examiner has suggested to further define methods for targeted recombination in which the TFO has a Kd of $2 \times 10^{-7}$ or less. Therefore, the claims as amended are enabled by the specification.

(2) Claim 15 has been amended to further define the method of claim 7 to produce changes in the genome of an intact human or animal further containing the steps of injecting the oligonucleotide into an intact human or animal having a sequence that forms a triple-stranded nucleic acid molecule with the target sequence located in the genome of the intact human or animal, wherein the oligonucleotide binds to the targeted sequence with a Kd of less than or equal to $2 \times 10^{-7}$, and mutates the target sequence. The examiner at page 8, lines 3-5 of the office action, states that the specification discloses that in mice (***in vivo***) mutagenesis was observed in liver, skin, kidney, colon, small intestine, and lung cells at a frequency five fold that of background (Example 7). In addition, Example 1 at pages 15-27 describes targeted mutagenesis by TFOs ***in vivo*** of monkey COS cells, patient derived XPA cells, patient-derived XPV cells, and normal human fibroblasts. While, as stated above, no examples are required, the specification clearly enables the method of targeted recombination to produce changes in the genome of an intact human or animal. See also the more recent papers by the applicant that further demonstrate that TFOs can produce inheritable changes in intact animals and animal cells, which, even though these occur at a very low frequency, are sufficient to treat disease. See, for example, Faruqi, et al., Mol. Cell. Biol. 20(3):990-1000 (2000); Seidman and Glazer, J. Clin. Invest. 112(4):487-494 (2003); and Luo, et al.,Proc. Natl. Acad. Sci. USA 97(16):9003-9008 (2000). It is important to note that although the examiner's recognize that delivery is an issue, it

is also established that it is not insurmountable and evidence shows that it is possible to practice the claimed methods.

In contrast to the examiner's statements, the key finding of Wang Mol. Cellular Biol. 15(3):1759-1768, 1767 (1995)) is that the binding affinity of TFO's to the target site as measured *in vitro*, was highly correlated with their **intracellular** activity. This work showed that psoralen-conjugated TFOs transfected into monkey COS cells can induce base pair-specific mutations within the supF mutation reported gene in a simian virus 40 (SV 40) genome in these cells. Chan, J. Biol. Chem. 274:11541-11548 (1999) discloses that tethered donor-TFOs (TD-TFOs) mediate targeted sequence alterations within a SV40 shuttle vector in mammalian cells. They observed successful reversion of the supF target gene after *in vitro* co-incubation of the target vector with the TD-TFOs and also after an ***in vivo*** protocol in which cells already containing the shuttle vector were transfected with the oligonucleotides (p. 11547). Barre Proc. Natl. Acad. Sci. 97; 3084-3088 (2000) discloses at page 8 that the results of their work demonstrate **without ambiguity** that TFOs are capable of modifying an **endogenous** target gene. While Seidman J. Clin. Invest. 112: 487-494 (2003) discloses that there are obstacles to TFO activity, Seidman also describes several ***in vivo*** studies demonstrating the ability of TFOs to induce targeted recombination (p. 490-491). In summary, the specification discloses that in mice (***in vivo***) mutagenesis was observed in liver, skin, kidney, colon, small intestine, and lung cells at a frequency five fold that of background following injection of a TFO (AG30) (Example 7) and discloses targeted mutagenesis by TFOs ***in vivo*** of monkey COS cells, patient derived XPA cells, patient-derived XPV cells, and normal human fibroblasts (Example 1). One does not have to do

breeding studies to show inheritance. Inheritance can be shown by replication of modified cells, wherein the progeny cells also show the modification. See the attached definitions of inheritable ("This is the "internally coded, inheritable information" carried by all living organisms. This stored information is used as a "blueprint" or set of instructions for building and maintaining a living creature. These instructions are found within almost all cells (the "internal" part), they are written in a coded language (the genetic code), they are copied at the time of cell division or reproduction and are passed from one generation to the next ("inheritable"). These instructions are intimately involved with all aspects of the life of a cell or an organism. They control everything from the formation of protein macromolecules, to the regulation of metabolism and synthesis."). Subsequent papers provide further support for the statements and methods described in the application, and evidence that low frequency recombination is sufficient for efficacy. The fact that some experimentation is required does not mean that the claims are not enabled. Therefore, the claims as amended are enabled by the specification.

**Rejection Under 35 U.S.C. § 112, second paragraph**

A. Claims 7-12 and 15-25 were rejected under 35 U.S.C. § 112, second paragraph, as indefinite for allegedly not defining the steps of providing a single-stranded molecule and providing a donor nucleic acid resulting in targeted recombination. Applicants respectfully traverse this rejection to the extent that it is applied to the claims as amended.

Claim 7 has been amended to further define the method for targeted recombination of a nucleic acid molecule as containing the steps of (1) providing a single-stranded oligonucleotide having a sequence that forms a triple-stranded nucleic acid molecule by hybridizing with a

target sequence double-stranded nucleic acid molecule with a Kd of less than or equal to 2 x 10-7, and (2) providing a donor nucleic acid **such that recombination of the donor nucleic acid into the target sequence is induced by triple helix formation between the single-stranded oligonucleotide and the double-stranded nucleic acid molecule**. Support for this amendment can be found in the specification at least at page 7, lines 12-18. Claim 7 as amended defines that the triplex formation between the single-stranded oligonucleotide and the target sequence double-stranded nucleic acid molecule stimulates recombination of a donor nucleic acid into the target sequence. It should be noted that the only steps performed by a person are providing the oligonucleotides and the target. The oligonucleotides by definition hybridize and effect the modification. Therefore, claims 7-12 and 15-25 as amended are not indefinite.

B. Claims 9, 12, and 25 were rejected under 35 U.S.C. § 112, second paragraph, as indefinite. Applicants respectfully traverse this rejection to the extent that it is applied to the claims as amended.

Claims 9, 12, and 25 have been amended to correct antecedent basis. These claims as amended recite a "donor nucleic acid" as recited in the claims upon which claims 9, 12, and 25 depend. Therefore, claims 9, 12, and 25 as amended are not indefinite.

C. Claims 15-24 were rejected under 35 U.S.C. § 112, second paragraph, as being indefinite. Applicants respectfully traverse this rejection to the extent that it is applied to the claims as amended.

Claim 15 has been amended to further define the method of claim 7 to produce changes in the genome of an intact human or animal further containing the steps of **administering** the

oligonucleotide into an intact human or animal having a sequence that forms a triple-stranded nucleic acid molecule with the target sequence located in the genome of the intact human or animal, wherein the oligonucleotide binds to the targeted sequence with a Kd of less than or equal to $2 \times 10^{-7}$, and mutates the target sequence. Claim 15 as amended defines the relationship between the oligonucleotide of claim 7 and the oligonucleotide of claim 15. Claim 15 as amended clarifies what the oligonucleotide is injected into and refers to the target sequence as recited in claim 7. Finally, claims 15-24 as amended further define the method of targeted recombination as defined by claim 7 by administering the oligonucleotide into an intact human or animal such that the oligonucleotide mutates the target sequence. Therefore, claims 15-24 as amended are not indefinite.

D. Claims 19 and 20 were rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for use of the term "DNA fragment". Applicants respectfully traverse this rejection to the extent that it is applied to the claims as amended.

Claims 19 and 20 have been amended to recite the term "donor nucleic acid" as recited in the claims upon which claims 19 and 20 depend. Therefore, claims 19 and 20 are not indefinite.

**Priority**

The present application is a continuation-in-part of U.S.S.N. 09/411,291 filed on October 4, 1999, which is a divisional of U.S.S.N. 08/476,712 filed on June 7, 1995, page 1, paragraph 1. U.S.S.N. 09/411,291, which issued as U.S. Patent No. 6,303,376 ("the '376 patent"), and U.S.S.N. 08/476,712, which issued as U.S. Patent No. 5,962,426 ("the '426 patent"), provide support under 35 U.S.C. 120 for the claims of the present application.

The discussion that follows refers to the '376 patent. The disclosure of the '376 patent and the '426 patent is the same since the 1999 application which issued as the '376 patent is a divisional of the 1995 application which issued as the '426 patent and contains no new subject matter. (1) The claims define methods of targeted recombination using a TFO in combination with a tethered or unlinked donor nucleic acid. The '376 patent discloses at column 3, lines 1-4 that "the binding of the oligonucleotide to the target region stimulates mutations within or adjacent to the target region using cellular DNA synthesis, **recombination**, and repair mechanisms." The '376 patent also discloses that TFO's are particularly useful as a tool to cause targeted mutagenesis. Those of ordinary skill in the art will recognize that mutagenesis can be caused by **recombination**. Therefore, targeted mutagenesis includes targeted recombination. Furthermore, the '376 patent discloses at column 3, lines 49-56, that TFOs can be used to stimulate recombination of a DNA fragment into a target region, but does not distinguish between whether the DNA fragment is linked or unlinked. However, at column 1 to column 2, the '376 patent discloses methods of use of TFOs alone or linked to reactive moieties. The '376 patent also discloses at column 6, lines 40-58, that TFOs can be used to stimulate recombination of a DNA fragment administered in combination with the TFO into a target region. Furthermore the '376 patent discloses at column 6, lines 31-39, that the TFOs can be used alone or in combination and gives as an example a psoralen-**linked** oligonucleotide. Therefore, the '376 patent discloses that TFOs can be used to stimulate recombination administered in combination with a DNA fragment, the '376 patent discloses that the TFOs can be used alone or in combination, and the '376 patent discloses that TFOs can be linked to reactive moieties. It

would be obvious to one of skill in the art that the DNA fragment could be unlinked or linked to the TFO. Therefore, the claims as amended of the present application are supported by the disclosure of the '376 and '426 patents.

(2) The claims define a TFO with a Kd of $2 \times 10^{-7}$ or less. Support for these claims can be found in the '376 patent at least at column 5, lines 3-4, and at column 9, lines 25-56, and at Table 1. Therefore, the claims as amended of the present application are supported by the disclosure of the '376 and '426 patents.

(3) The claims further define the method for targeted recombination as defined by claim 7 to produce changes in the genome of an intact human or animal that contains the steps of injecting the oligonucleotide into an intact human or animal that binds to the target sequence and mutates the target sequence. The '376 patent describes methods of producing changes in the genome of a human or animal in the specification at least at column 2, lines 11-59, and at column 2, lines 6-41, respectively. Furthermore, the '376 patent discloses at least at column 5, lines 49-58, that the oligonucleotides are preferably injected into mammals. As mentioned above, the '376 patent discloses at least at column 3, lines 49-56, and again at column 6, lines 40-58, methods in which the TFOs can be used to stimulate homologous recombination of a DNA fragment into a target region. Therefore, the claims of the present application are supported by the disclosures of the '376 and '426 patents.

The present application differs from the earlier filed application primarily by virture of the examples. Example 1, which is found in the '376 and '426 patents as well as the present application, specifically describes targeted mutagenesis by TFOs ***in vivo*** of monkey COS cells,

patient derived XPA cells, patient derived XPV cells, and normal human fibroblasts. The remaining examples, 2 through 8, found only in the present application, support the findings disclosed in example 1. Example 2 describes the ability of TFOs to promote recombination in human cell-free extracts. Examples 3, 4, and 5 describe the role of recombination and repair proteins in the pathway of TFO induced recombination. Example 6 describes targeted mutagenesis by TFOs at genomic sites in somatic cells of adult mice. Example 7 describes heritable changes produced by TFO induced recombination in adult mice. Example 8 describes that induced mutagenesis is specifically brought about through triple-helix formation.

It should also be noted that these examples were submitted in the prosecution of the parent application in the form of a 132 declaration by the applicant, in order to overcome very similar 112 rejections. It was clearly made of record in the prior prosecuted application that the examples were supportive of disclosure; not adding new subject matter.

Therefore, the claims of the present application are supported by the disclosures of the '376 and '426 patents.

**Rejections Under 35 U.S.C. § 102 and 103**

Claims 7-12, 15-21, 23-25 were rejected under 35 U.S.C. § 102(b) as being anticipated by Chan, et al., ("Chan") J. Biol. Chem. 274: 11541-11548 (1999)). Claim 22 was rejected under 35 U.S.C. § 103(a) as being unpatentable over Chan et al.. Applicants respectfully traverse these rejections to the extent that it is applied to the claims as amended.

As discussed above, the present application is entitled to a priority date of 1995. This application is fully entitled to priority under 35 U.S.C. 120 for the claimed subject matter. Therefore, Chan is not available as prior art.

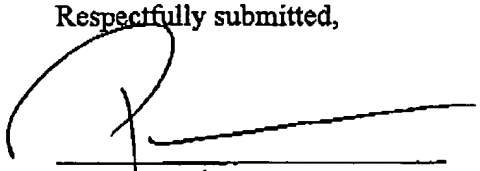
**Double Patenting Rejection**

Claims 7, 8, 10-12, 15-21, 23, and 24 were rejected under the judicially created doctrine of obviousness-type double patenting as being unpatentable over claims 1-34 of U.S. Patent No. 5,776,744 to Glazer et al ("Glazer"). Claims 9, 22, and 25 were rejected under the doctrine of obviousness-type double patenting over claims 1-34 of Glazer in combination with Chan.

U.S. Patent No. 5,776,744 expired on July 8, 2002 for failure to pay the maintenance fee. A copy of the patent bibliographic data as listed by the U.S. P.T.O. is enclosed for your convenience. Therefore, this rejection is moot.

Allowance of claims 7-12 and 15-25 as amended is respectfully solicited.

Respectfully submitted,



Patrea L. Pabst
Reg. No. 31,284

Date: March 1, 2005

PABST PATENT GROUP LLP
400 Colony Square, Suite 1200
1201 Peachtree Street
Atlanta, Georgia 30361
(404) 879-2151
(404) 879-2160 (Facsimile)

Return to:

USPTO Home Page

Finance Online Shopping Page

United States Patent and Trademark Office

| Patent Bibliographic Data | | | | | 11/16/2004 01:26 P |
|---|---|---|---|---|---|
| Patent Number: | 5776744 | | Application Number: | 08467126 | |
| Issue Date: | 07/07/1998 | | Filing Date: | 06/07/1995 | |
| Title: | METHODS AND COMPOSITIONS FOR EFFECTING HOMOLOGOUS RECOMBINATION | | | | |
| Status: | Expired for non-payment on: 07/08/2002 | | | Entity: | Small |
| Window Opens: | 07/09/2001 | Surcharge Date: | 01/08/2002 | Expiration: | 07/08/20 |
| Fee Amt Due: | $0.00 | Surchg Amt Due: | $0.00 | Total Amt Due: | $0.00 |
| Fee Code: | | | | | |
| Surcharge Fee Code: | | | | | |
| Most recent events (up to 7): | 2002/07/08<br>2002/01/30 | Patent Expired for Failure to Pay Maintenance Fees.<br>Maintenance Fee Reminder Mailed.<br>— End of Maintenance History — | | | |
| Address for fee purposes: | BURNS DOANE SWECKER & MATHIS L L P<br>POST OFFICE BOX 1404<br>ALEXANDRIA, VA<br>223131404 | | | | |

**NOTE: All USPTO fees are subject to change. If you are making a payment please check the www.uspto.g website or contact the Maintenance fee branch (703-308-5068) to confirm the amount due on the date payment is to be made. A maintenance fee payment can be timely made using the certificate of mailing or transmission procedure set forth in 37 CFR 1.8.**

Run Another Query

Need Help? | USPTO Home Page | Finance Online Shopping Page

# Definition


**... the outward physical manifestation of internally coded, inheritable, information.**

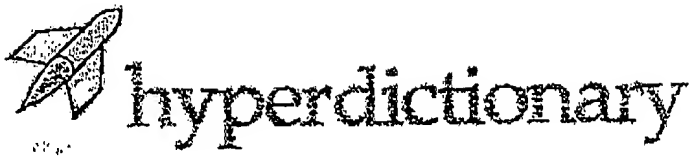
**There are two parts to this definition ...**

**Phenotype** This is the "outward, physical manifestation" of the organism. These are the physical parts, the sum of the atoms, molecules, macromolecules, cells, structures, metabolism, energy utilization, tissues, organs, reflexes and behaviors; anything that is part of the observable structure, function or behavior of a living organism.

**Genotype** This is the "internally coded, inheritable information" carried by all living organisms. This stored information is used as a "blueprint" or set of instructions for building and maintaining a living creature. These instructions are found within almost all cells (the "internal" part), they are written in a coded language (the genetic code), they are copied at the time of cell division or reproduction and are passed from one generation to the next ("inheritable"). These instructions are intimately involved with all aspects of the life of a cell or an organism. They control everything from the formation of protein macromolecules, to the regulation of metabolism and synthesis.

**Return to :**

Science at a Distance ---- Biological Information ---- Genotype and Phenotype






# Meaning of INHERITABLE

Pronunciation: in'heritubul

**WordNet Dictionary**

**Definition:** [adj] that can be inherited; "inheritable traits such as eye color"; "an inheritable title"

**Websites:** 

**Synonyms:** ancestral, familial, genetic, hereditary, heritable, inherited, inheriting, monogenic, nee, patrim polygenic, transmissible, transmitted

**Antonyms:** nonheritable, noninheritable

**Webster's 1913 Dictionary**

**Definition:** \In*her"it*a*blo\, a.
1. Capable of being inherited; transmissible or descendible; as, an inheritable estate or title. --Blackstone.

2. Capable of being transmitted from parent to child; as, inheritable qualities or infirmities.

3. [Cf. OF. enheritable, inheritable.] Capable of taking by inheritance, or of receiving by descent; capable of succeeding to, as an heir.

> By attainder . . . the blood of the person attainted is so corrupted as to be rendered no longer inheritable. --Blackstone.
>
> The eldest daughter of the king is also alone inheritable to the crown on failure of issue male. --Blackstone.

[Inheritable blood], blood or relationship by which a person becomes qualified to be an heir, or to transmit possessions by inheritance.

# High-frequency intrachromosomal gene conversion induced by triplex-forming oligonucleotides microinjected into mouse cells

Zhongjun Luo, Margaret A. Macris, A. Fawad Faruqi, and Peter M. Glazer*

Departments of Therapeutic Radiology and Genetics, Yale University School of Medicine, P.O. Box 208040, New Haven, CT 06520-8040

Edited by Charles M. Radding, Yale University School of Medicine, New Haven, CT, and approved June 5, 2000 (received for review January 6, 2000)

To test the ability of triple helix-forming oligonucleotides (TFOs) to promote recombination within chromosomal sites in mammalian cells, a mouse LTK$^-$ cell line was established carrying two mutant copies of the herpes simplex virus thymidine kinase (TK) gene as direct repeats in a single chromosomal locus. Recombination between these repeats can produce a functional TK gene and occurs at a spontaneous frequency of $4 \times 10^{-6}$ under standard culture conditions. When cells were microinjected with TFOs designed to bind to a 30-bp polypurine site situated between the two TK genes, recombination was observed at frequencies in the range of 1%, 2,500-fold above the background. Recombination was induced efficiently by injection of both psoralen-conjugated TFOs (followed by long-wave UVA light; 1.2%) and unconjugated TFOs alone (1.0%). Control oligomers of scrambled sequence but identical base composition were ineffective, and no TFO-induced recombination was seen in a control LTK$^-$ cell line carrying an otherwise identical dual TK gene construct lacking the 30-bp polypurine target site. TFOs transfected with cationic lipids also induced recombinants in a highly sequence-specific manner but were less effective, with induced recombination frequencies of 6- to 7-fold over background. Examination of the TFO-induced recombinants by genomic Southern blotting revealed gene conversion events in which both TK genes were retained, but either the upstream (57%) or the downstream gene (43%) was corrected to wild type. These results suggest that, with efficient intracellular delivery, TFOs may be effective tools to promote site-specific recombination and targeted modification of chromosomal loci.

gene targeting | recombination | thymidine kinase

Genetic manipulation of mammalian cells has been a major research tool. Gene transfer methods coupled with techniques to select for cells in culture that have undergone site-specific homologous recombination have facilitated specific gene replacement and enabled the development of genetically altered "knock-out" mice (1). These selection methods improve the apparent efficiency of gene targeting but do so by eliminating clones arising from nonhomologous events. They do not alter the absolute frequency of homologous recombination, which is typically low in mammalian cells in gene transfer experiments (2). This low frequency of homologous recombination limits the extension of this technology to gene therapy, and therefore efforts have been made to improve the efficiency of gene targeting.

A series of studies have focused on modification of the recipient chromosomal site to create a substrate prone to homologous recombination. The site-specific endonuclease I-*Sce*I can induce double-strand breaks (DSBs) within both extrachromosomal and chromosomal DNA loci engineered to carry the rare 18-bp recognition site (3). Such targeted DSBs have been shown to boost substantially the frequency of intramolecular and intermolecular recombination in mammalian cells and also in *Xenopus* oocytes (4–8). However, this approach requires the prior introduction of the recognition site within the genome.

In addition to DSBs, DNA damage from UV light, alkylating agents, and photoreactive molecules such as psoralen has been shown to be recombinagenic (9–11) but in a non-site-specific way. However, in previous work, we found that site-specific DNA damage could be introduced in mammalian cells by taking advantage of the sequence specificity of oligonucleotide-mediated triple helix formation (12–15). Triplex DNA can be formed when oligonucleotides bind in the major groove of the double helix in a sequence-dependent manner at polypurine/polypyrimidine stretches in duplex DNA (reviewed in ref. 16). The specificity arises from the base triplets formed by either Hoogsteen or reverse-Hoogsteen hydrogen bonding between the third strand and the purine strand of the duplex. In previous studies, we demonstrated that psoralen-conjugated triplex-forming oligonucleotides (TFOs) could mediate the introduction of base pair-specific psoralen adducts (and consequently mutations) in mammalian cells (12).

Using this strategy, we found that triple helix-targeted psoralen photoadducts could induce recombination within a simian virus 40-based shuttle vector carrying two mutant copies of the *supF* reporter gene (17). In addition, prompted by data showing that intermolecular triple helix formation, even in the absence of covalent DNA damage, could provoke DNA repair (13), we also tested the ability of non-psoralen-conjugated TFOs to induce recombination in the episomal simian virus 40 target. We found that third strands capable of high-affinity binding to the target DNA were able to stimulate recombination in a pathway that depended on nucleotide excision repair (NER; ref. 18).

These results raised the possibility that high-affinity TFOs, with or without an associated DNA reactive conjugate, might serve as tools to sensitize a *chromosomal* site to recombination. To investigate the feasibility of such an approach, we established a mouse LTK$^-$ cell line carrying two mutant copies of the herpes simplex virus thymidine kinase (TK) gene as direct repeats in a single chromosomal locus. In this construct, recombination can be detected by reconstruction of a functional TK gene. Using a series of oligonucleotides, we performed experiments showing that high-affinity TFOs, with or without psoralen, are capable of stimulating recombination between the tandem TK genes. Experiments in which oligonucleotides were transfected into cells by cationic lipids demonstrated that the induced recombination depended on the specificity of the TFO for the target locus but yielded modest levels of induction. In contrast, intranuclear delivery of TFOs by direct microinjection produced recombinants at frequencies greater than 1%, 2,500- to 3,000-fold over background and in the range of the best results in model systems employing I-*Sce*I for DSB generation. Analysis of the TFO-induced recombinant clones was consistent with a pathway of homology-directed gene conversion. The results

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: TFO, triplex-forming oligonucleotide; TK, thymidine kinase; NER, nucleotide excision repair; DSB, double-strand break; kb, kilobase.

*To whom reprint requests should be addressed. E-mail: peter.glazer@yale.edu.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Article published online before print: *Proc. Natl. Acad. Sci. USA*, 10.1073/pnas.160004997. Article and publication date are at www.pnas.org/cgi/doi/10.1073/pnas.160004997

suggest that, with effective delivery, TFOs can be potent agents for promoting homologous recombination at targeted chromosomal sites.

## Materials and Methods

**Oligonucleotides.** Unconjugated and psoralen-linked oligonucleotides were synthesized by the Keck Facility at Yale University by using standard phosphoramidite chemistry and materials from Glen Research (Sterling, VA). All oligomers contained phosphodiester backbones and were synthesized to contain a 3′ propylamine group to minimize susceptibility to degradation by 3′ exonucleases (19). The oligomers were purified by either gel electrophoresis or HPLC, followed by Centricon-3 filtration in distilled water (Amicon). The psoralen was incorporated into the oligonucleotide synthesis as a psoralen phosphoramidite, resulting in an oligonucleotide linked at its 5′ end via a six-carbon linker arm to 4′-hydroxymethyl-4,5′,8-trimethyl psoralen. The oligonucleotides used in this study were pso-AG30, (5′ psoralen-AGGAAGGGGGGGGTGGTGGGGGAGGGGGAG-3′), AG30 (same as pso-AG30 but without 5′ psoralen), pso-SCR30 (5′ psoralen-GGAGGAGTGGAGGGGAGTGAGGGGGGG-GGG-3′), and SCR30.

**Plasmids.** Plasmid pJS-3, containing two mutant copies of the herpes simplex virus TK gene along with the neomycin-resistance gene, was obtained from M. Liskay (Oregon Health Sciences University, Portland, OR; ref. 20). The TK genes in pJS-3 contain *Xho*I linker insertion mutations at positions 735 (TK26) and 1,220 (TK8). They are present as 2.0-kilobase (kb) and 2.5-kb fragments in direct repeat orientation in the *Hin*dIII and *Bam*HI sites in the vector, respectively (Fig. 1). A 200-bp fragment carrying the *supFG1* gene from the plasmid pSupFG1 (12) was amplified by PCR with primers incorporating *Cla*I recognition sites and was inserted into the unique *Cla*I site in pJS-3 situated in the middle of the 1.3-kb stretch between the TK genes, yielding the plasmid pTK2supF (Fig. 1). The *supFG1* gene contains a 30-bp G-rich polypurine site that affords high-affinity triple helix formation in the antiparallel purine motif by the TFO AG30.

**Cells.** Mouse LTK$^-$ cells were obtained from the American Type Culture Collection and were grown in DMEM supplemented with 10% (vol/vol) FBS. The LTK$^-$ cells were transfected with 20 ng of plasmid pTK2supF linearized at the *Hpa*II site by using Lipofectamine (Life Technologies, Bethesda, MD) as directed by the manufacturer. Selection for transfectants was carried out in 400 μg/ml G418 in DMEM. Transfectants were analyzed for integrated plasmid structure and copy number by Southern analysis of genomic DNA as described (20). The clone designated FL-10, determined to contain a single copy of the pTK2supF construct (data not shown), was chosen for further study. The LTK$^-$-derivative cell line pJS-3-10, containing a single copy of the original pJS-3 vector (lacking the *supFG1* polypurine target site), was obtained from M. Liskay (21) and was used as a control.

**Microinjection of Oligonucleotides and Recombination Assay.** A day before microinjection, selected LTK$^-$-derived cell lines (either FL-10 or pJS-3-10) were seeded at a density of 375 cells per cm$^2$ in 30-mm dishes on which grids consisting of 25 squares were drawn. One cell at the top left of each square was microinjected. The injection needles were pulled with micropipette puller model P-87 (Sutter Instruments, Novato, CA) from 1.2/0.94-mm (outside diameter/inside diameter) borosilicate capillaries with filaments (World Precision Instruments, Sarasota, FL). The cells were microinjected with an Eppendorf 5170 micromanipulator and an Eppendorf 5242 microinjector equipped with Zeiss Axiovert 135 microscope. Solutions of selected oligonucleotides at 8 μM were injected in volumes of ≈15 fl directly into cell nuclei to deliver an estimated 72,000 oligonucleotides per injection, yielding an intranuclear concentration in the range of $2 \times 10^{-7}$ to $2 \times 10^{-6}$ M, depending on the precise number of molecules injected and the actual nuclear volume.

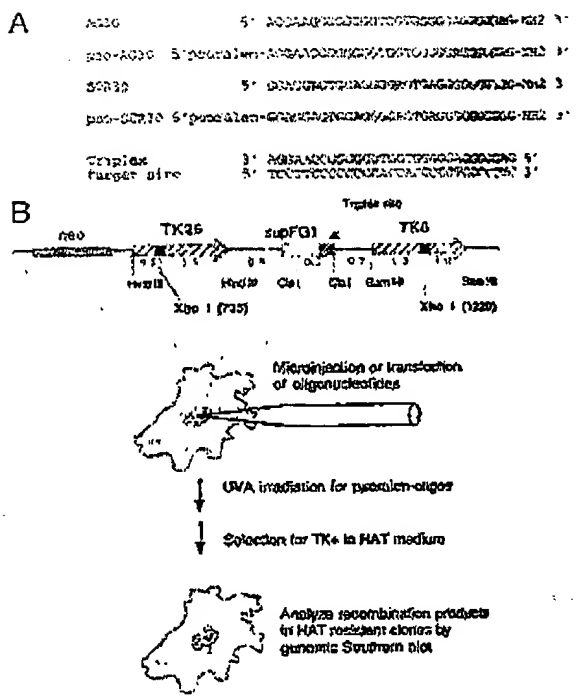



**Fig. 1.** Experimental scheme to investigate induction of intrachromosomal recombination by TFOs. LTK$^-$ cells carrying, at a single chromosomal locus, two mutant copies of the TK gene as direct repeats flanking a polypurine third-strand binding site were used to test the ability of transfected or microinjected TFOs to promote recombination. A purine-rich oligonucleotide of length 30 (AG30) was designed to form a triple helix in the antiparallel triplex motif at the G-rich target site, as shown. As a control, Scr30, containing the same base composition but a scrambled sequence, was used. In some experiments, the AG30 and the SCR30 oligonucleotides were conjugated at their 5′ ends to 4′-hydroxymethyl-4,5′,8-trimethylpsoralen via the 4′-hydroxymethyl position. In this case, by formation of the triple helix, psoralen intercalation and photoaddition is targeted to the thymidines at the predicted duplex-triplex junction. Potential recombinants are identified as TK$^+$ clones growing in selective HAT ($1 \times 10^{-4}$ M hypoxanthine/$2 \times 10^{-6}$ M aminopterin/$1.6 \times 10^{-5}$ M thymidine)-containing medium.

**Transfection with Cationic Lipids.** Cells at a density of $1.67 \times 10^4$ per cm$^2$ ($1 \times 10^6$ in 100-mm dishes) were transfected with 10 μg of oligonucleotide DNA per dish mixed with 66 μl of GenePorter and diluted into a total of 2 ml of serum-free medium, as directed by the manufacturer (Gene Therapy Systems, San Diego). In the case of the psoralen-conjugated oligomers, UVA irradiation was given 5 h after transfection, after which the cells were placed in full growth medium supplemented with 10% (vol/vol) FBS. Medium was changed to HAT selection 24 h later.

**Southern Blot Analyses.** HAT-resistant colonies representing candidate TK$^+$ recombinant clones were identified after 2 weeks. The

Table 1. Intrachromosomal recombination induced by TFOs

| Cells | Oligonucleotide | Transfection method | UVA | TK+ clones/total cells | Frequency, % |
|---|---|---|---|---|---|
| FL-10 | None | None | | 4/1 × $10^6$ | 0.0004 |
| | None | Cationic lipids | + | 40/6 × $10^6$ | 0.0007 |
| | pso-Scr30 | Cationic lipids | + | 44/6 × $10^6$ | 0.0007 |
| | AG30 | Cationic lipids | | 149/6 × $10^6$ | 0.0025 |
| | pso-AG30 | Cationic lipids | + | 178/6 × $10^6$ | 0.0030 |
| | pso-Scr30 | Microinjection | + | 0/1,375 | ≤0.072 |
| | AG30 | Microinjection | | 14/1,375 | 1.0 |
| | pso-AG30 | Microinjection | + | 16/1,375 | 1.2 |
| pJS-3-10 | None | None | | 12/2 × $10^5$ | 0.0006 |
| | pso-AG30 | Microinjection | + | 0/650 | ≤0.15 |

clones were expanded and genomic DNA was isolated by using Genzyme genomic DNA purification kits as described by the supplier. Genomic DNA was subject to restriction by either *Bam*HI and *Hin*dIII or *Bam*HI, *Hin*dIII, and *Xho*I (New England Biolabs). Southern analysis was performed by using standard methods by gel electrophoresis through 0.9% agarose and transfer to Hybond-N membranes (Amersham Pharmacia), with the $^{32}$P-labeled 2.5-kb *Bam*HI TK fragment from pTK2supF used as a probe.

**Sequence Analysis of Genomic DNA.** A 200-bp region flanking the polypurine triplex target site was amplified from the genomic DNA of selected HAT-resistant clones by PCR. Primers used were JS3-P6 (5′-CATGACATTAACCTATAAAAATAGGCG-3′) and JS3-P7 (5′-GGTTAAGTCCTCATTTAAATTAGGCA-3′). An internal primer, JS3-P11 (5′-TTAAATTAGGCAAAG-GAATTC-3′) was used for DNA sequence analysis via automated methods (22).

## Results

**Experimental Strategy.** To investigate TFO-induced recombination at a chromosomal site, we established a mouse LTK$^-$ cell line subclone (FL-10) carrying a pair of mutant TK genes in a single locus as direct repeats separated by 1.3-kb (Fig. 1). In this construct, the region between the TK genes was engineered to contain a 30-bp G-rich polypurine sequence (present in the *supFG1* gene insert in the *Cla*I site) that previous work had shown to be a site amenable to high-affinity third-strand binding in the antiparallel triplex motif (23) by the TFO designated AG30 (ref. 12; Fig. 1). The TK genes contain inactivating *Xho*I linker insertion mutations at different sites (positions 735 in TK 26 and 1,220 in TK8). The TK26 and TK8 genes are present as 2.0-kb and 2.5-kb fragments within *Hin*dIII and *Bam*HI sites, respectively. The stable integration of this construct into the LTK$^-$ cells and its presence in a single copy was confirmed by Southern analysis of genomic DNA from the FL-10 subclone (data not shown).

In the assay, recombination between the two TK genes has the potential to produce a functional gene. Because the parental LTK$^-$ cells lack the cellular TK, cells in which the mutant TK genes have recombined to generate a wild-type TK can be selected by growth in the presence of HAT medium. Induction of recombination by selected oligonucleotides is quantified by enumerating the HAT-resistant colonies as a proportion of the total number of cells treated.

For comparison, a similar LTK$^-$ cell line obtained from M. Liskay was also used (21). This line, pJS-3-10, contains a single chromosomally integrated copy of a dual TK construct similar to that in FL-10 but lacks the *supFG1* gene insert. Hence, in the pJS-3-10 cells, the recombination substrate lacks the 30-bp polypurine target site for AG30. Also, in FL-10 cells, TK26 is upstream of TK8, whereas in pJS-3-10 cells, TK8 is upstream of TK26, with polarity defined with respect to the direction of TK gene transcription. Previous work by Liskay and colleagues (21) showed that the TK8 and TK26 genes individually revert to wild type at frequencies in the range of $10^{-8}$ or lower and thus generation of a functional TK gene at a measurable frequency requires information transfer between the two genes. In the pJS-3-10 cells, recombination between the TK genes occurs at spontaneous frequencies in the range of $10^{-6}$ (21).

**TFO-Induced Intrachromosomal Recombination.** The FL-10 and pJS-3-10 cell lines were used to test the ability of TFOs to stimulate intrachromosomal recombination in a site-specific manner. The cells were transfected with a series of oligonucleotides by two different methods (either comixture with cationic lipids or direct intranuclear microinjection), and the production of HAT-resistant colonies expressing wild-type TK was determined.

The oligonucleotides used are shown in Fig. 1 and included both psoralen-conjugated oligomers as well as unconjugated ones (with the only modification in the latter case being 3′ end protection with a 3′ propylamine group to provide nuclease resistance). The TFOs specific for the polypurine site between the two TK genes, AG30 and pso-AG30, were determined in previous work to bind with high-affinity to the duplex target site, with equilibrium dissociation constants of 1 × $10^{-8}$ M and 3 × $10^{-9}$ M, respectively (12, 13). The control oligomers, Scr30 and pso-Scr30, have the same G-rich base composition as the AG30 TFOs but in a scrambled sequence (creating 14 mismatches of 30 in the third-strand binding code for the polypurine target site versus only 2 mismatches in the case of AG30). These oligomers show no detectable binding to the 30-bp target site even at concentrations up to $10^{-5}$ M in gel mobility-shift assays (unpublished results). In the case of the psoralen-conjugated oligomers, UVA irradiation (1.8 J/cm$^2$) was given to the cells after the transfections to photoactivate the psoralen for potential photoadduct formation at the target site. The timing of the UVA irradiation differed depending on the transfection method. The microinjected cells were irradiated 1 h after injection, whereas the cells transfected with cationic lipids were irradiated 5 h after addition of the oligonucleotide/lipid mixtures.

The results (Table 1) show that only AG30 and pso-AG30 were effective in inducing HAT-resistant clones in the FL-10 cells at frequencies substantially above the background level of 4 × $10^{-6}$ in the untreated cells. However, the efficiency of induction by the specific TFOs varied significantly depending on the method of TFO delivery. Transfection of the FL-10 cells with AG30 and pso-AG30 (plus UVA light) by using cationic lipids yielded recombinants at frequencies of 25 × $10^{-6}$ and 30 × $10^{-6}$, respectively, values 6- and 7-fold above the background in untreated cells. Remarkably, microinjection of AG30 and pso-AG30 produced HAT-resistant colonies at frequencies of 1.0% and 1.2%, 2,500- and 3,000-fold above background. These values were calculated as the ratio of the number of HAT-resistant colonies produced divided by the total number of cells injected with the oligonucleotides. In the microinjection procedure, a volume of 15 fl of a solution containing 8 μM oligonucleotide was injected into the nuclei, yielding approximately

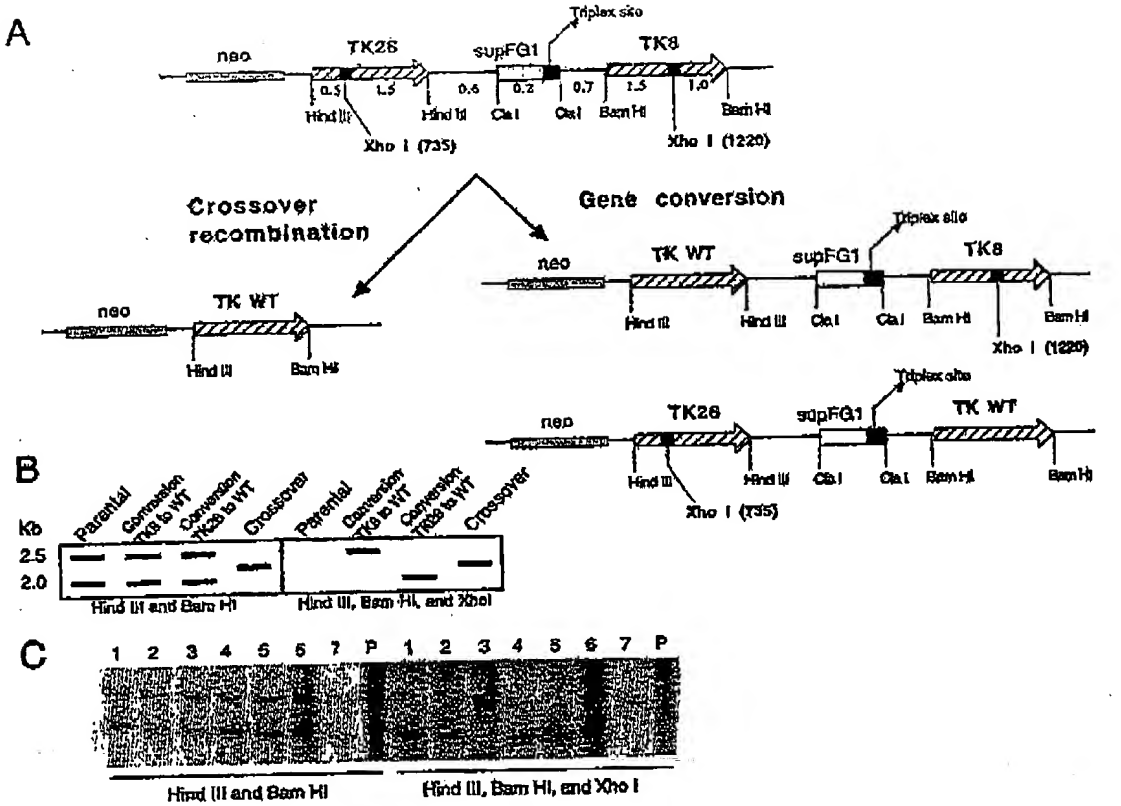



Fig. 2. Analysis of TFO-induced recombinant clones expressing wild-type (WT) TK. (*A*) Possible pathways to generate a wild-type TK gene from the tandem mutant TK genes in FL-10 cells. The diagram illustrates crossover recombination, yielding (via multiple crossovers) a single wild-type TK gene in a nonconservative event, and gene conversion, in which there is information transfer from one TK gene to the other to correct the *Xho*I linker insertion mutation, with retention of both TK genes in a conservative event. (*B*) Expected pattern of bands on Southern analysis of parental and recombinant HAT-resistant clones, depending on the nature of the recombination event. The hypothetical band pattern is based on the indicated restriction digestion of genomic DNA and hybridization with a TK gene fragment as a probe. (*C*) Southern blot analysis of genomic DNA from the parental FL-10 cells (P) and seven HAT-resistant clones produced by TFO microinjection (lanes 1–7). The samples were restricted with the indicated enzymes, and the Southern hybridization was performed with the 2.5-kb TK gene *Bam*HI fragment as a probe.

72,000 oligonucleotide molecules per nucleus. Cell viability after microinjection was estimated to be ≈70%, based on experiments that used the same protocol to inject a green fluorescent protein expression vector instead of the TFOs (data not shown). However, this viability estimate was not used to correct the induced recombination frequencies, because we do not know exactly the post-injection viability in the oligonucleotide experiments.

In comparison, microinjection of pso-Scr30 into the FL-10 cells, followed by UVA irradiation, yielded no HAT-resistant clones (Table 1). As an additional control, pso-AG30 was also microinjected into the pJS-3-10 cells under the same conditions as with the FL-10 cells. The pJS-3-10 cells, like the FL-10 cells, are an LTK$^-$ subclone. They contain a dual TK construct similar to the one in the FL-10 cells, with the only differences being the absence of the polypurine target site and the order of the TK8 and TK26 alleles as they reside in the chromosome (21). As shown (Table 1), no recombinants were detected in 650 injected cells.

**Analysis of HAT-Resistant Clones.** The HAT-resistant clones induced by microinjection of FL-10 cells with AG30 ($n = 14$) and pso-AG30 ($n = 16$) were isolated, recloned, and expanded. Genomic DNA was prepared for Southern analysis to examine the structure of the TK locus and to determine the nature of the recombination products.

Possible recombination pathways that can generate a functional TK gene are illustrated in Fig. 2*A*. A nonconservative event involving crossover recombination can produce a single copy of a wild-type TK gene. However, because of the orientation of the inactivating mutations in TK26 and TK8, a single crossover would generate a doubly mutant TK gene retained in the chromosome and would pop out the reconstructed wild-type TK gene. Multiple crossovers would be required for a nonconservative event to yield a wild-type allele in the chromosome. Alternatively, there can be information transfer from one TK gene to the other to correct the *Xho*I linker insertion mutation, as diagrammed, in a conservative process of gene conversion that retains both TK gene fragments, one now wild-type and one still mutant.

The expected pattern of bands on Southern analysis from each of these possible pathways is illustrated schematically in Fig. 2*B*. In the parental HAT-sensitive FL-10 cells, genomic DNA restricted with *Bam*HI and *Hin*dIII and probed with a TK gene fragment should yield two bands of 2.0 and 2.5 kb. These bands represent the 2.0-kb TK26 *Hin*dIII fragment and the 2.5-kb TK8 *Bam*HI fragment. Both of these bands will be eliminated by the addition of *Xho*I to the restriction digest, because both mutant TK fragments contain the *Xho*I linker insertions.

In the case of a nonconservative crossover recombinant, only a single band should be visualized in the *Bam*HI and *Hin*dIII lane; however, this band should persist when *Xho*I is added, because the recombinant fragment would contain the wild-type TK sequence without either of the *Xho*I sites.

Table 2. Intrachromosomal recombination products induced by TFOs

| Oligonucleotide treatment | Frequency of TK+ colonies, % | Gene conversion | | Crossover recombination |
|---|---|---|---|---|
| | | TK8 to WT | TK26 to WT | |
| pso-AG30 + UVA | 1.2 (16/1,375) | 9/16 (56%) | 7/16 (44%) | 0/16 (0%) |
| AG30 | 1.0 (14/1,375) | 8/14 (57%) | 6/14 (43%) | 0/14 (0%) |

WT, wild type.

The gene conversion events can involve either correction of TK8 to wild type or correction of TK26 to wild type. In the former case, the 2.0-kb and 2.5-kb bands will be present on double digestion, but the 2.0-kb band will be lost in the triple digestion (because the *Xho*I linker insertion remains in the TK26 gene). The 2.5-kb band will remain, because the gene conversion event would eliminate the *Xho*I site in the TK8 gene. With conversion of TK26 to wild type, again both bands will be present in the *Bam*HI and *Hin*dIII double digest lane, but in this case, the 2.5-kb band will be lost with addition of *Xho*I, and the 2.0-kb band will be resistant to *Xho*I and thus will persist in the three-enzyme sample.

An example of this analysis performed on one series of recombinant clones induced by pso-AG30 is shown in Fig. 2*C*. All seven clones analyzed in this blot showed two bands on *Bam*HI and *Hin*dIII digestion. With the addition of *Xho*I, clones 1, 2, 4, 5, and 6 showed loss of the 2.5-kb band and retention of the 2.0-kb one, whereas, conversely, clones 3 and 7 showed loss of the 2.0-kb band and persistence of the 2.5-kb one. These results are consistent with all seven clones having arisen from gene conversion events, with 1, 2, 4, 5, and 6 being convertants from TK26 to wild type and 3 and 7 resulting from gene conversion from TK8 to wild type.

Table 2 presents a summary of the results of the Southern analysis. All 14 HAT-resistant clones induced by AG30 and all 16 induced by pso-AG30 were found to have arisen by gene conversion. In both groups, the distribution of gene conversion events was almost identical, with 43% TK26 converting to wild type and 57% TK8 converting to wild type for the AG30-induced events and 44% and 56% for pso-AG30-induced events, respectively.

**Analysis of the Triple Helix Target Site.** In previous work, we had found that triple helix formation by TFOs can cause mutations at and around the target site (13). We therefore examined the putative third-strand binding site and surrounding sequences in randomly selected TFO-induced, HAT-resistant recombinants to determine whether the target region had undergone mutation at the same time that recombination had been stimulated. For each of 21 HAT-resistant clones tested, a 200-bp region of genomic DNA encompassing the polypurine/polypyrimidine site was amplified by PCR, and the DNA sequence of the region was determined. No mutations were seen in any of the clones. These results indicate that triplex-induced recombination is not necessarily accompanied by mutation of the third-strand binding site. However, a low frequency of TFO-induced mutagenesis cannot be ruled out. Also, the HAT-selection assay specifically identifies cells in which a functional TK gene has been generated; cells with other classes of nonparental products are not detected, and some of these might contain target site mutations.

## Discussion

Results obtained with a mouse LTK cell line carrying two mutant copies of the TK gene as direct repeats in a single chromosomal locus show that oligonucleotides designed to bind as third strands to a site between the genes can effectively stimulate intrachromosomal recombination. When the TFOs were introduced into the cells by intranuclear microinjection, the frequency of induced recombination was in the range of 1%, 2,500-fold greater than the background frequency in untreated cells. By Southern analysis of the genomic DNA from the nonparental TK+ colonies, the TFO-induced recombination products were found to have arisen from conservative gene conversion events in which one mutant gene was corrected via information transfer from the other. In addition, although triplex formation can induce mutations as well as recombination (13), no mutations were seen in or around the predicted TFO binding site in the triplex-induced recombinants.

Interestingly, the AG30 TFO itself, even without conjugation to a DNA-reactive molecule, was almost as effective as the psoralen-conjugated version, pso-AG30 (1.0% versus 1.2% when both were microinjected and $25 \times 10^{-6}$ versus $30 \times 10^{-6}$, respectively, when the TFOs were transfected with cationic lipids). These comparisons suggest two possibilities. (*i*) The yield of targeted photoadducts was low, possibly because the protocol for psoralen-TFO transfection and the timing of the UVA irradiation were not optimal. Hence, the results obtained with pso-AG30 may reflect primarily the effect of the triple helix formation alone. (*ii*) Targeted photoadducts were formed, but the TFO-mediated triplexes themselves are nearly as recombinagenic as the third-strand targeted psoralen adducts. Distinguishing between these possibilities will require further analyses, including direct measurements of third-strand-directed photoadduct formation at the target locus (24, 25). Nonetheless, the activity of the AG30 TFO by itself in promoting recombination demonstrates that TFOs capable of high-affinity, site-specific binding to DNA, even without the generation of covalent damage, can provoke DNA metabolism and thereby stimulate recombination.

The induced recombination frequencies achieved on microinjection of the TFOs (1% to 1.2%) are in the same range as results reported in experiments that used similar tandem repeat gene targets in which site-specific DSBs are produced within the target locus by expression of the I-*Sce*I nuclease in the cells. For example, Taghian and Nickoloff (26) and Donoho *et al.* (27) observed I-*Sce*I-induced recombination between neo gene repeats at frequencies of 1% and 3% in CHO and mouse embryonic stem cells, respectively. Although the recombination substrates in these studies were not exactly the same as in our work and although the I-*Sce*I cleavage was targeted to sites within one of the duplicated genes rather than between them, this comparison nonetheless suggests that TFOs, with effective intranuclear delivery, can induce recombination at a chromosomal target with an efficiency rivaling that of a site-specific endonuclease.

In other experiments that provide a model for targeted gene replacement, Smih *et al.* (5) showed that, in cells with a single copy of the neo gene interrupted by an I-*Sce*I recognition site insert, expression of the I-*Sce*I nuclease could sensitize the target to recombination with a transfected neo gene fragment, at a frequency at least 50-fold above background. In a similar experimental design, Donoho *et al.* (27) also demonstrated the utility of I-*Sce*I in promoting targeted recombination of a transfected DNA with a chromosomal locus containing an I-*Sce*I site, observing targeting frequencies of up to 1%, 5,000-fold higher than in the absence of I-*Sce*I expression. These experiments showed that induced strand breaks at a chromosomal locus can have a substantial effect on homologous recombination between the locus and an exogenously introduced DNA.

The experiments presented herein suggest that triplex formation can provoke sufficient DNA metabolism to stimulate *intra*molecular homologous recombination at a chromosomal locus at high efficiency. By analogy to the I-*Sce*I data, we hypothesize that TFOs

may also prove to be effective reagents for promoting *intermolecular* recombination between a transfected gene fragment and a chromosomal target site.

However, the comparison to the I-*Sce*I data does not necessarily imply that the TFOs mediate recombination by producing the same DSB type of damage as I-*Sce*I. At this point, we have not performed experiments to examine directly the pattern of triplex-stimulated strand breaks. However, previous work has suggested that triple helix formation creates an altered DNA structure that is recognized by the NER complex in mammalian cells (13). It was found that triplexes formed on a plasmid substrate can stimulate DNA repair activity, as measured by DNA repair synthesis, in human cell extracts (13). Such repair activity is absent from extracts of cells deficient in the NER damage recognition factor XPA (unpublished results). The generation of repair synthesis tracts in plasmids to which the third strands have bound implies the generation of at least one single-strand break, which would be the minimum damage necessary to enable the incorporation of labeled nucleotides. Theoretically, if the canonical NER reaction (28) were carried out on the triplex-containing plasmid, a pattern of dual endonuclease incisions flanking the triple helix would be expected. The current data from the repair synthesis assay do not allow us to distinguish between the production of a single nick, followed by nick translation, and the generation of two nicks and a consequent gap, followed by gap-filling repair synthesis. However, if DSBs in the plasmids were produced at a significant frequency in the extracts, we would expect to see linearized DNA molecules, and no such species have been seen (ref. 13 and unpublished results).

At this point, we favor the possibility that one or more single-strand breaks are produced at or near the TFO-binding site via repair-directed endonuclease incision activity. However, the number, position, and strand orientation of the possible repair-mediated strand breaks remain to be determined. Also, the role of the NER pathway in the TFO-induced *intrachromosomal* gene conversion reported herein has not been tested explicitly. However, by extrapolation from our recent work demonstrating a requirement for NER in TFO-stimulated recombination in an *episomal* simian virus 40-based shuttle vector (18), we speculate that stimulation of recombination by triplex formation at a chromosomal site will also turn out to require NER activity.

The recombination products produced by the TFOs in the present work were all consistent with conservative gene conversion events. However, the manner in which the dual TK recombination substrate resides in the genome of the FL-10 cells favors the detection of gene conversion events. Detectable nonconservative events would require multiple crossovers and would therefore be rare. Single crossover events stimulated by triplex formation would yield doubly mutant TK genes and would not be detected in the assay. Hence, the results reported herein may underestimate the ability of TFOs to provoke intrachromatid recombination.

The ability of TFOs to stimulate recombination at a chromosomal locus demonstrates that chromatin is not an absolute barrier to third-strand binding to chromosomal DNA, consistent with recent studies that have detected TFO-directed mutagenesis at chromosomal sites (14, 15). However, in the mutagenesis studies, the TFOs were delivered into the cells by electroporation, passive fluid phase uptake, or cationic lipids, and the induced mutation frequencies, although above background, were in the $10^{-4}$ to $10^{-3}$ range. In the current work, a key finding is that microinjection of the TFOs yielded induced recombination frequencies several orders of magnitude greater than when the TFOs were introduced by co-mixture with cationic lipids. These results show that the biological obstacles to TFO-mediated genome modification include not only the chromatin structure of the target gene but also the cellular barriers that must be traversed by the TFOs to achieve an effective concentration in the appropriate intranuclear compartment for binding to the target site.

In our experiments, we estimate that intranuclear concentrations in the range of $2 \times 10^{-7}$ to $2 \times 10^{-6}$ M were achieved. These values are similar to those reported for oligomers transfected by other means, and thus the improved efficacy of the microinjection may be due to a more biologically effective intranuclear distribution of the TFOs. However, although microinjection is feasible as a research tool, its applicability for gene therapy is limited. Hence, facile techniques for effective intranuclear oligonucleotide delivery are needed to make this strategy more practical.

Nonetheless, the results reported herein support the utility of TFOs as tools to sensitize genomic sites to recombination. Although our experiments focused on recombination within a specially engineered chromosomal locus containing a tandem gene repeat, this strategy should be applicable to the stimulation of recombination between a chromosomal site and exogenously introduced DNA molecules. As such, this work may facilitate efforts directed at gene replacement or correction for research or clinical purposes. One current limitation is that triplex formation occurs predominately at polypurine/polypyrimidine sites. However, a considerable effort has been directed at extending the third-strand binding code (16), and in any case, polypurine sites are overrepresented in the genome. In addition, the present findings raise the possibility that not just TFOs but also other high-affinity DNA-binding molecules, such as peptide nucleic acids (29) and polyamides (30), may prove useful in strategies to promote site-specific recombination for the purpose of genome modification.

We thank M. Seidman and D. Carroll for helpful discussions and P. Chan, J. Yuan, S. Shiao, K. Vasquez, L. Narayanan, R. Franklin, S. J. Baserga, and L. Cabral for their help. This work was supported by National Institutes of Health Grant RO1 GM54731 and by a Scholar Award to P.M.G. from the Leukemia and Lymphoma Society. Z.L. was supported by National Institutes of Health Training Grant T32 CA09159. A.F.F. was supported in part by the Anna Fuller Foundation.

1. Capecchi, M. R. (1989) *Science* 244, 1288–1292.
2. Hanson, K. D. & Sedivy, J. M. (1995) *Mol. Cell. Biol.* 15, 45–51.
3. Rouet, P., Smih, F. & Jasin, M. (1994) *Mol. Cell. Biol.* 14, 8096–8106.
4. Inbar-Sovlich, T., Yang, D. & Waldman, A. S. (1994) *Nucleic Acids Res.* 22, 5649–5657.
5. Smith, F., Rouet, P., Romanienko, P. J. & Jasin, M. (1995) *Nucleic Acids Res.* 23, 3012–5019.
6. Choulika, A., Perrin, A., Dujon, B. & Nicolas, J. F. (1995) *Mol. Cell. Biol.* 15, 1968–1973.
7. Brenneman, M., Gimble, F. S. & Wilson, J. H. (1996) *Proc. Natl. Acad. Sci. USA* 93, 3608–3612.
8. Segal, D. J. & Carroll, D. (1995) *Proc. Natl. Acad. Sci. USA* 92, 806–810.
9. Bhattacharyya, N. P., Maher, V. M. & McCormick, J. J. (1989) *Mutat. Res.* 211, 205–214.
10. Tsujimura, T., Maher, V. M., Godwin, A. R., Liskay, R. M. & McCormick, J. J. (1990) *Proc. Natl. Acad. Sci. USA* 87, 1566–1570.
11. Vos, J. M. & Hanawalt, P. C. (1989) *Mol. Cell. Biol.* 9, 2897–2905.
12. Wang, G., Levy, D. D., Seidman, M. M. & Glazer, P. M. (1995) *Mol. Cell. Biol.* 15, 1759–1768.
13. Wang, G., Seidman, M. M. & Glazer, P. M. (1996) *Science* 271, 802–805.
14. Majumdar, A., Khorlin, A., Dyatkina, N., Lin, F.-L. M., Powell, J., Liu, J., Pei, Z., Khripine, Y., Watanabe, K. A., George, J., Glazer, P. M. & Seidman, M. M. (1998) *Nat. Genet.* 20, 212–214.
15. Vasquez, K. M., Wang, G., Havre, P. A. & Glazer, P. M. (1999) *Nucleic Acids Res.* 27, 1176–1181.
16. Chan, P. P. & Glazer, P. M. (1997) *J. Mol. Med.* 75, 267–282.
17. Faruqi, A. F., Seidman, M. M., Segal, D. J., Carroll, D. & Glazer, P. M. (1996) *Mol. Cell. Biol.* 16, 6820–6828.
18. Faruqi, A. F., Datta, H. J., Carroll, D., Seidman, M. M. & Glazer, P. M. (2000) *Mol. Cell. Biol.* 20, 990–1000.
19. Zentgut, J. G., Vasquez, K. M., Tinsley, J. H., Kessler, D. J. & Hogan, M. E. (1992) *Nucleic Acids Res.* 20, 307–314.
20. Liskay, R. M. & Stachelek, J. L. (1986) *Proc. Natl. Acad. Sci. USA* 83, 1802–1806.
21. Letsou, A. & Liskay, R. M. (1987) *Genetics* 117, 759–769.
22. Havre, P. A., Gunther, E. J., Gasparro, F. P. & Glazer, P. M. (1993) *Proc. Natl. Acad. Sci. USA* 90, 7879–7883.
23. Beal, P. A. & Dervan, P. B. (1991) *Science* 251, 1360–1363.
24. Giovannangeli, C., Diviacco, S., Labrousse, V., Gryaznov, S., Charneau, P. & Helene, C. (1997) *Proc. Natl. Acad. Sci. USA* 94, 79–84.
25. Oh, D. H. & Hanawalt, P. C. (1999) *Nucleic Acids Res.* 27, 4734–4742.
26. Taghian, D. G. & Nickoloff, J. A. (1997) *Mol. Cell. Biol.* 17, 6386–6393.
27. Donoho, G., Jasin, M. & Berg, P. (1998) *Mol. Cell. Biol.* 18, 4070–4078.
28. Sancar, A. (1996) *Annu. Rev. Biochem.* 65, 43–81.
29. Faruqi, A. F., Egholm, M. & Glazer, P. M. (1998) *Proc. Natl. Acad. Sci. USA* 95, 1398–1403.
30. White, S., Szewczyk, J. W., Turner, J. M., Baird, E. E. & Dervan, P. B. (1998) *Nature (London)* 391, 468–471.

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 21, Issue of May 25, pp. 18018–18025, 2001
Printed in U.S.A.


# Triplex-induced Recombination in Human Cell-free Extracts

## DEPENDENCE ON XPA AND HsRad51*

Received for publication, December 22, 2000, and in revised form, February 20, 2001
Published, JBC Papers in Press, February 27, 2001, DOI 10.1074/jbc.M011646200

**Hirock J. Datta, Phillip P. Chan, Karen M. Vasquez, Ravindra C. Gupta, and Peter M. Glazer‡**

*From the Departments of Therapeutic Radiology and Genetics, Yale University School of Medicine, New Haven, Connecticut 06520-8040*

**Triple helix-forming oligonucleotides (TFOs) can bind to polypurine/polypyrimidine regions in DNA in a sequence-specific manner. Triple helix formation has been shown to stimulate recombination in mammalian cells in both episomal and chromosomal targets containing direct repeat sequences. Bifunctional oligonucleotides consisting of a recombination donor domain tethered to a TFO domain were found to mediate site-specific recombination in an intracellular SV40 vector target. To elucidate the mechanism of triplex-induced recombination, we have examined the ability of intermolecular triplexes to provoke recombination within plasmid substrates in human cell-free extracts. An assay for reversion of a point mutation in the *supFG1* gene in the plasmid pSupFG1/G144C was established in which recombination in the extracts was detected upon transformation into indicator bacteria. A bifunctional oligonucleotide containing a 30-nucleotide TFO domain linked to a 40-nucleotide donor domain was found to mediate gene correction *in vitro* at a frequency of 46 × $10^{-5}$, at least 20-fold above background and over 4-fold greater than the donor segment alone. Physical linkage of the TFO to the donor was unnecessary, as co-mixture of separate TFO and donor segments also yielded elevated gene correction frequencies. When the recombination and repair proteins HsRad51 and XPA were depleted from the extracts using specific antibodies, the triplex-induced recombination was diminished, but was either partially or completely restored upon supplementation with the purified HsRad51 or XPA proteins, respectively. These results establish that triplex-induced, intermolecular recombination between plasmid targets and short fragments of homologous DNA can be detected in human cell extracts and that this process is dependent on both XPA and HsRad51.**

Targeted modification of the genome by gene replacement is of value as a research tool and has potential application to gene therapy. However, although facile methods exist to introduce new genes into mammalian cells, the frequency of homologous integration is limited (1), and isolation of cells with site-specific gene insertion typically requires a selection procedure (2). Site-specific DNA damage in the form of double-strand breaks produced by rare cutting endonucleases can promote homologous recombination at chromosomal loci in several cell systems (3–7), but this approach requires the prior insertion of the recognition sequence into the locus. Because intermolecular triple helices can provoke DNA repair (8), oligonucleotide-mediated triple helix formation has been proposed as a potentially more general approach to sensitizing a target site to homologous recombination (9–12).

TFOs[1] can bind in the major groove of DNA to polypurine/polypyrimidine sequences, forming specific Hoogsteen or reverse-Hoogsteen hydrogen bonds with the purine strand of the duplex (13, 14). Triplex formation has been shown to inhibit transcription in mammalian cells (15) and can be used to deliver a DNA-reactive conjugate to a specific target site both in complex DNA mixtures *in vitro* (16, 17) and within mammalian cells in culture (18–22), in some cases leading to site-directed mutations (19, 20). Triplex formation, by itself, can be mutagenic, and evidence suggests that the nucleotide excision repair (NER) and transcription-coupled repair pathways may play a role in the triplex-induced mutagenesis (8).

In previous work, we found that triple helix-directed psoralen cross-links could stimulate recombination in a plasmid substrate containing two tandem copies of the *supFG1* reporter gene (9). Subsequent work established that triplex formation, even in the absence of covalent DNA damage, could stimulate recombination between repeated sequences, an effect that was absent in cells deficient in the NER factor, XPA (10). Recent work has extended these findings to the demonstration of TFO-induced recombination at a chromosomal locus containing two tandem copies of the herpes simplex virus thymidine kinase gene, following direct intranuclear microinjection of the oligonucleotides (11).

Based on the ability of TFOs to mediate specific molecular recognition of a DNA target site within a cellular genome and on the observation that triplex formation can stimulate recombination, we also tested a series of bifunctional oligonucleotides consisting of a TFO designed to bind to bp 167–196 of the *supFG1* reporter gene coupled to a short (40 nt) segment of DNA homologous to bp 121–160 of the gene. Such a hybrid molecule, designated a tethered donor-TFO (TD-TFO), was found to mediate recombination with the *supFG1* gene present in an SV40-based episomal vector in monkey cells at frequencies in the range of 0.1–1% (Ref. 23 and data not shown), demonstrating that TFOs can promote *inter*molecular as well as *intra*molecular recombination in mammalian cells. This result is consistent with studies demonstrating that bifunctional

* This work was supported by National Institutes of Health Grants RO1GM54781 and RO1CA64186 (to P. M. G.) and R37GM33504 (to C. M. R.) and by a scholar award (to P. M. G.) from the Leukemia and Lymphoma Society. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Depts. of Therapeutic Radiology and Genetics, Yale University School of Medicine, P.O. Box 208040, New Haven, CT 06520-8040. Tel.: 203-737-2788; Fax: 203-737-2630; E-mail: peter.glazer@yale.edu.

[1] The abbreviations used are: TFO, triple helix-forming oligonucleotide; NER, nucleotide excision repair; nt, nucleotide(s); bp, base pair(s); DTT, dithiothreitol; TD, tethered donor; HPLC, high pressure liquid chromatography.

oligonucleotides can mediate both triplex formation and strand invasion on plasmid substrates *in vitro* (24, 25).

In the present study, we have used a plasmid-based assay to investigate triple helix-induced recombination in human cell-free extracts. We find that triple helix formation can stimulate recombination between a plasmid and short homologous fragments *in vitro*. Stimulation was observed whether or not the donor fragment was directly linked to the TFO. Recombination was reduced in the absence of the TFO as well as when the TFO was substituted with a non-triplex-forming, scrambled sequence oligonucleotide. To probe the mechanism of the induced recombination, the roles of the NER damage recognition factor, XPA (26), and the human recombinase, HsRad51 (27), were directly tested by experimental manipulation of the respective protein levels in the extracts, either via immunodepletion with specific antibodies or supplementation with purified proteins. We report here that both XPA and HsRad51 are required for triple helix-induced recombination, and that increased HsRad51 levels can boost the efficiency of the reaction.

## EXPERIMENTAL PROCEDURES

*Plasmid Vector*—The shuttle vector plasmid pSupFG1/G144C, containing a *supFG1* gene with an inactivating G:C to C:G point mutation at position 144, was described previously (23).

*Oligonucleotides*—Oligonucleotides were synthesized by the Midland Certified Reagent Co. (Midland, TX) and purified by either gel electrophoresis or high pressure liquid chromatography (HPLC), followed by Centricon-3 filtration in distilled water (Amicon, Beverly, MA). The oligonucleotides consisted primarily of phosphodiester linkages but were modified at the 3′ end to resist exonuclease activity by the inclusion of phosphorothioate linkages at the terminal three residues. In the TD-TFO molecule (designated A–AG30), the linker segment between the donor fragment and the TFO domain consisted of the sequence 9TT9TT9, in which 9 indicates a 9-atom polyethylene glycol linker (Spacer 9, Glen Research, Sterling, VA). The specific TFO, designated AG30, has the sequence 5′-AGGAAGGGGGGGGTGGTGGGGGAGGGGAAG-3′ and is designed to bind as a third strand to bp 167–176 of the *supFG1* gene. The donor domain (A) consists of a 40-nt synthetic single-stranded DNA fragment homologous to positions 121–160 of the *supFG1* gene (5′-AGGGAGCAGACTCTAAATCTGCCGTCATCGACTTCGAAGG-3′). The scrambled sequence oligonucleotide, SCR30, has the same base composition as AG30 but differs at 12 positions: 5′-GGAGGAGTGGAGGGGAGTGAGGGGGGGGGG-3′.

*Cells*—Construction of *E. coli* SY302 *lacZ125*(Am) *recA56 hsdR2*::Tn*10* trp-49 has been described previously (28). HeLa cells were maintained and grown by the National Cell Culture Center (Minneapolis, MN) and were obtained as cell pellets for extract preparation.

*Proteins and Antibodies*—HsRad51 protein was purified from *E. coli* DH10B (Life Technologies, Inc.) carrying plasmid pEG932. Purification consisted of chromatography through Q Sepharose, Bio-Gel-http, Mono-Q, and native DNA-cellulose. Other purification details have been reported previously (29). Purification was documented by SDS-polyacrylamide gel electrophoresis analysis, yielding a single visualized band of 37 kDa following the DNA-cellulose purification step. Purified HsRad51 protein was injected into rabbits to produce high affinity polyclonal antibodies specific to the HsRad51 protein (29).

XPA protein was produced using an *Escherichia coli* expression vector (obtained from R. Wood) containing the human XPA cDNA sequence, along with an N-terminal 6-histidine tag, in the pET-15b plasmid (Novagen, Madison, WI). The protein was expressed in an *E. coli* expression strain, BL21(DE3) pLysS, as described by Jones and Wood (30). Following expression, XPA was purified using immobilized metal-affinity chromatography (Talon resin; CLONTECH, Palo Alto, CA) under native conditions. Fractions were eluted with a buffer containing 20 mM Tris, pH 8.0, 100 mM NaCl, and 100 mM imidazole. Western analysis was used to determine specificity using antibodies to the 6-His tag. Coomassie staining was used to determine purity. To further confirm that the expressed and purified protein was the correct species and present as soluble monomers in solution, XPA protein was subjected to both mass spectrometry and HPLC size exclusion chromatography/laser light scattering analysis. The results revealed a monodispersed peak at a molecular mass of 36.8 kDa, indicating a monomer of the correct size. Rabbits were immunized with the purified XPA protein (100 μg/injection) to produce high affinity antibodies specific to XPA.

*Preparation of Cell-free Extract*—HeLa whole cell extract was prepared as described previously (28). Briefly, HeLa cells were washed with phosphate-buffered saline and resuspended in 0.01 N Tris-HCl, pH 7.9, 1 mM EDTA, 5 mM DTT, followed by lysis using a Dounce homogenizer. The lysate was diluted in four packed cell volumes of 0.05 M Tris-HCl, pH 7.9, 0.01 M $MgCl_2$, 2 mM DTT, 25% sucrose, 50% glycerol, and a protease inhibitor mixture (Sigma catalog no. P8340). One packed cell volume of saturated $(NH_4)_2SO_4$ (0.33 g/ml of solution) was added and then neutralized by 1 N NaOH, followed by centrifugation at 15,000 × *g* for 20 min at 4 °C. The pellet was resuspended in 0.025 M HEPES, pH 7.9, 0.1 N KCl, 0.012 N $MgCl_2$, 0.05 mM EDTA, 2 mM DTT, 17% glycerol and was dialyzed in the same buffer for 8–12 h. The sample was quick frozen in liquid $N_2$ and stored at −80 °C. The preparation typically contained 15–20 mg of protein/ml.

*In Vitro Assay for Recombination*—Reactions consisted of 3 μg of pSupFG1/G144C plasmid DNA, 3 μg each of selected oligonucleotides (TFO, donor fragment, or both), 60 mM NaCl, 2 mM β-mercaptoethanol, 3 mM KCl, 12 mM Tris-HCl, pH 7.4, 2 mM ATP, 0.1 mM each dNTPs, 2.5 mM creatine phosphate, 1 μg of creatine phosphokinase, 12 mM $MgCl_2$, 0.1 mM spermidine, 2% glycerol, 0.2 mM DTT, and 15–20 μl of cell-free extract in a 50-μl total reaction volume. After incubating 2 h at 30 °C, the reactions were terminated by the addition of 25 μM EDTA, 0.5% SDS, and 20 μg of proteinase K. After incubation at 37 °C for 1 h, the plasmid DNA was isolated by phenol extraction and ethanol precipitation and dissolved in 10 μl of $H_2O$. 1 μl of the resulting sample was used to transform *E. coli* SY302 by electroporation, as described (28), followed by growth of the cells on indicator plates for genetic analysis of *supFG1* gene function as described previously (19).

*Depletion of HsRad51 and XPA from Cell Extracts*—Anti-HsRad51 or anti-XPA sera were adjusted to 1× Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 100 mM NaCl) and then incubated with pre-swollen Protein A-Sepharose beads for 1 h at 4 °C. The beads were washed three times with Tris-buffered saline buffer and incubated with 50 μl of HeLa cell extract for 2 h on ice with gentle rotation. The supernatant (HsRad51- or XPA-depleted extract) was recovered by centrifugation and subsequently examined by Western blot and used in the *in vitro* recombination assay.

*Solubilization of RAD51 Immunoprecipitates*—After incubating the cell extract with HsRad51 antibody-Protein A-Sepharose beads, the beads were centrifuged and washed twice with 10 mM phosphate buffer, pH 7.2. Then one bead volume of 100 mM glycine, pH 2.5, and 3.5 M $MgCl_2$ was added and kept at 4 °C. After 1 h, the sample was centrifuged and the supernatant was immediately dialyzed against a buffer containing 20 mM potassium phosphate, pH 7.4, 0.5 mM DTT, 0.2 mM EDTA, 10% glycerol, and 100 mM KCl. The supernatant was directly used to supplement the depleted extracts for the recombination assay without freezing.

## RESULTS

*Experimental Design*—The substrate for triplex-targeted recombination was the plasmid vector pSupFG1/G144C, containing a mutated version of the *supFG1* amber suppressor tRNA gene, *supFG1*-144, which has an inactivating G:C to C:G mutation at bp 144. The function of this gene can be readily assayed in indicator bacteria carrying an amber stop codon in the *lacZ* gene, and so *supFG1*-144 is a useful reporter of recombination events that revert the gene to the functional sequence. The *supFG1*-144 gene also contains a 30-bp, G-rich site at the 3′ end of the gene to which the G-rich 30-mer TFO (AG30) can bind to form a triple helix in the anti-parallel motif (Fig. 1).

In a strategy to promote targeted recombination, we designed a TD-TFO molecule (A–AG30) in which the AG30 TFO is tethered to a donor DNA fragment homologous to a region of the *supFG1*-144 target gene via a mixed sequence linker (Fig. 1) (23). This arrangement facilitates target site recognition via triple helix formation while at the same time positioning the donor fragment for possible recombination and information transfer. This strategy also is intended to exploit the ability of a triple helix, itself, to provoke DNA repair, potentially increasing the probability of recombination with the homologous donor DNA. In the bifunctional A–AG30 molecule, the donor fragment, A, consists of a single-strand of length 40 synthesized to be homologous to positions 121–160 of the *supFG1*-144 gene

except at position 144, where the sequence matches that of the functional *supFG1* gene.

*Triplex-induced Recombination in HeLa Cell-free Extracts*—In previous work, we demonstrated the occurrence of triplex-induced recombination upon transfection of A–AG30 into monkey COS cells already carrying the pSupFG1/G144C vector as an episomal, SV40-replicon-based target (23). To investigate the mechanism of triplex-induced recombination, in the present work we have tested the ability of triplex formation to promote recombination within human cell-free extracts.

Selected oligonucleotides were incubated with the target pSupFG1/G144C vector in HeLa whole cell extracts supplemented with nucleotides and ATP. Following a 2-h incubation, the plasmid vector DNA was isolated and used to transform *recA*, *lacZ*(Amber) indicator *E. coli* to score for *supFG1* gene function (Fig. 2). The results show that the bifunctional oligonucleotide, A–AG30, was active in the extracts and produced *supFG1*-144 gene reversion at a frequency of $45 \times 10^{-5}$. Note that this effect occurred in the extract and was not mediated by recombination in the indicator bacteria because, without incubation in the extract, no recombinant products were observed upon transformation of the A–AG30 sample into bacteria. The A donor fragment was also somewhat active, as co-mixture of A plus the pSupFG1/G144C plasmid led to a low level of *supFG1* reversion, consistent with the ability of short fragments of DNA to mediate recombination and marker rescue (31–33). However, the effect of A–AG30 was 4-fold higher than that of A alone, demonstrating the influence of the TFO domain and providing direct evidence for triplex-induced recombination *in vitro*. By itself, however, the TFO domain produced minimal reversion over background, indicating the need for the sequence information provided by the A donor fragment.

Interestingly, the sample in which AG30 and the A donor oligonucleotide were not linked but were simply co-mixed as separate molecules together with the plasmid substrate also produced an increased level of recombination, at a frequency of $40 \times 10^{-5}$, almost as high as that produced by the linked A–AG30. This result provides further evidence that a TFO can stimulate recombination between a donor fragment and a target locus. In addition, because the donor fragment in this case is separate from the TFO, the result specifically demonstrates a role for the TFO in stimulating recombination that is distinct from its ability to deliver a tethered donor fragment to the target site.

In another sample tested, the A donor was linked to an oligonucleotide segment designated SCR30, consisting of the same base composition as AG30 but a scrambled sequence creating 12 mismatches. SCR30 does not bind to the *supFG1* gene and so does not form a triplex. It also has no homology to the target gene. Linkage of SCR30 to the donor fragment was found to actually inhibit recombination relative to the donor fragment alone.

*Role of HsRad51 in Triplex-induced Recombination*—The results above establish that triplex-induced recombination can be reconstituted in HeLa cell extracts *in vitro*. By using this *in vitro* system, we sought to determine the role(s) of selected recombination and repair proteins in the pathway of triplex-induced recombination. HsRAD51 is a human recA homolog that functions in homologous recombination and has been shown to mediate DNA pairing and strand exchange reactions (34). To test the role of HsRAD51 protein in this homologous gene conversion, we used a polyclonal rabbit anti-HsRad51 antibody to deplete HsRad51 protein from the cell extract. Successful depletion of HsRad51 from the extract was confirmed by Western blot (Fig. 3). The depleted extract was tested for the ability to support triplex-induced recombination (Table I), both in the case of the linked A–AG30 bifunctional molecule and in the case of the co-mixed separate A and AG30 sample. Immunodepletion of HsRad51 was found to substantially reduce the frequency of recombinants in both cases. Control samples demonstrated that Protein A-Sepharose, in the absence of the HsRad51 polyclonal antibody, had no effect.

*Limited Complementation by Addition of Purified HsRad51 to the HsRad51-depleted Cell Extract*—Next, we tested the extent to which the triplex-induced recombination in the HsRad51-depleted extracts could be restored by the addition of

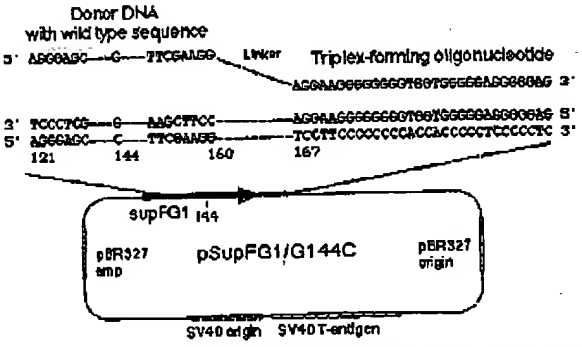



FIG. 1. **Schematic diagram depicting the binding of the AG30 TFO to the *supFG1*-144 gene in the vector pSupFG1/G144C.** AG30 in this example is linked to a 40-nt donor DNA fragment homologous to bp 121–160 of the *supFG1*-144 gene. The G at position 144 of the donor fragment is intended to correct the inactivating G:C to C:G mutation at bp 144 in the target gene. The linker segment consists of the sequence, 9TT9TT9, where "9" indicates a 9-atom polyethylene glycol linker. In some experiments, the donor fragment and the TFO were used separately or were co-mixed but not linked.

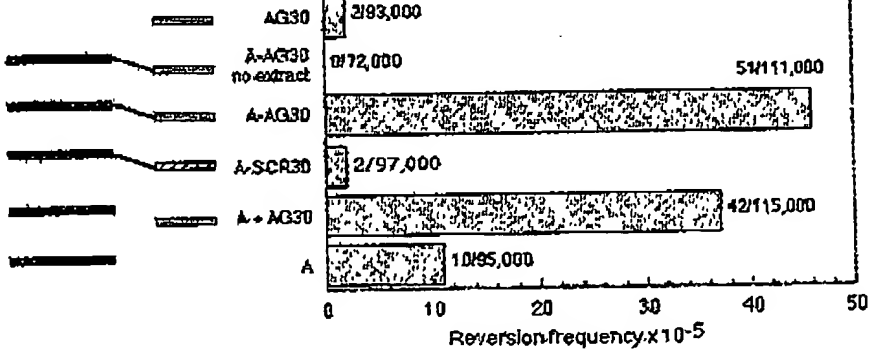



FIG. 2. **Triplex-induced recombination in human cell-free extracts.** The pSupFG1/G144C plasmid DNA was incubated *in vitro* with the indicated oligonucleotides in the presence or absence of HeLa whole cell extracts. After 2 h, the plasmid DNA was isolated and used to transform indicator bacteria for genetic analysis of the *supFG1* gene. A schematic diagram of each oligonucleotide or oligonucleotide combination is presented to the *left*. *Plus* sign (+) indicates that the different oligonucleotides are mixed together but unlinked. *Minus sign* (–) indicates that the various oligonucleotides are connected. The *bars* indicate the frequency of blue colonies (representing recombinants) out of the total colonies, with the actual count given to the *right* of each *bar*. The results are cumulative data from three experiments.

purified, recombinant HsRad51 protein. Increasing amounts of HsRad51 protein were added to the depleted extracts, and the recombination assay was carried out (Table II). Even after the addition of a large amount of HsRAD51 (up to 5 μg), only a portion of the triplex-induced recombination activity was recovered. We hypothesized that the lack of complementation by purified HsRad51 might reflect the removal from the immunodepleted extracts of other factors physically associated with HsRad51. To test this, we supplemented the immunodepleted extracts with re-solubilized HsRad51 immunoprecipitate (Table II). Addition of the solubilized immunoprecipitate to the depleted extracts was found to almost completely restore the recombination activity, indicating that HsRad51 immunodepletion removes more than HsRad51 alone and that HsRad51 supplementation, by itself, cannot compensate for the loss of the other factors. This result is not surprising in light of emerging evidence that the recombination complex in human cells consists of multiple factors, including Rad52, Rad54, XRCC2, and XRCC3, as well as members of the RAD51 family, including Rad51B/Rad51L1, Rad51C/Rad51L2, and Rad51D/Rad51L3 (35, 36).

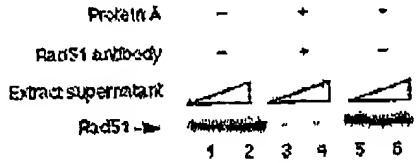



FIG. 3. **Immunodepletion of HsRad51 from the HeLa cell extracts.** Extract samples were immunodepleted using a polyclonal HsRad51 antibody pre-mixed with Protein A-Sepharose beads. The immunoprecipitate was removed by centrifugation, and the remaining supernatant was examined by Western blot analysis. Samples were treated as indicated.

*Addition of HsRad51 to the Complete Extract Boosts Activity*—Increasing amounts of HsRAD51 (from 250 ng to 2 μg) were added to non-depleted whole cell extracts, and triplex-induced recombination was measured (Table III). Both in the case of the linked donor fragment and TFO (A−AG30) and the unlinked donor plus TFO (A+AG30), additional HsRad51 was found to increase the frequency of the triplex-induced recombinants. In the samples supplemented with amounts of HsRad51 in the lower range, there was a minimal effect. However, at higher levels of supplementation, increased yields of recombinants

TABLE I

*Effect of HsRad51 depletion on triplex-induced recombination in cell-free extracts*

The results represent the combined data from three independent experiments.

| Oligonucleotide | Extract treatment | Blue colonies/total | Reversion frequency |
|---|---|---|---|
| | | | × $10^{-4}$ |
| None | Standard | 0/110,000 | 0 |
| A−AG30 | No extract | 0/95,000 | 0 |
| A−AG30 | Standard | 53/99,000 | 54 |
| A−AG30 | Protein A-Sepharose | 45/93,000 | 48 |
| A−AG30 | HsRad51 antibody | 9/110,000 | 8 |
| A+AG30 | Standard | 42/105,000 | 40 |
| A+AG30 | Protein A-Sepharose | 36/96,000 | 38 |
| A+AG30 | HsRad51 antibody | 9/99,000 | 9 |

TABLE II

*Effect of HsRad51 supplementation on triplex-induced recombination in HsRad51-depleted cell-free extracts*

The results represent the combined data from three independent experiments.

| Oligonucleotide | Extract treatment | Blue colonies/total | Reversion frequency |
|---|---|---|---|
| | | | × $10^{-4}$ |
| None | Standard | 0/111,000 | 0 |
| A−AG30 | No extract | 0/99,000 | 0 |
| A−AG30 | Standard | 51/112,000 | 46 |
| A−AG30 | HsRad51 antibody | 12/112,000 | 11 |
| A−AG30 | HsRad51 antibody + 500 ng of HsRad51 | 12/117,000 | 10 |
| A−AG30 | HsRad51 antibody + 1 μg of HsRad51 | 15/105,000 | 14 |
| A−AG30 | HsRad51 antibody + 2 μg of HsRad51 | 18/112,000 | 16 |
| A−AG30 | HsRad51 antibody + 5 μg of HsRad51 | 21/114,000 | 18 |
| A−AG30 | HsRad51 antibody + 10 μl of solubilized HsRad51 immunoprecipitate | 40/108,000 | 37 |
| A−AG30 | HsRad51 antibody + 20 μl of solubilized HsRad51 immunoprecipitate | 46/109,000 | 42 |

TABLE III

*Effect of HsRad51 supplementation on triplex-induced recombination in cell-free extracts*

The results represent the combined data from three independent experiments.

| Oligonucleotide | Extract treatment | Blue colonies/total | Reversion frequency |
|---|---|---|---|
| | | | × $10^{-4}$ |
| None | Standard | 0/93,000 | 0 |
| A−AG30 | No extract | 0/87,000 | 0 |
| A−AG30 | Standard | 54/110,000 | 49 |
| A−AG30 | Plus 250 ng of HsRad51 | 43/95,000 | 45 |
| A−AG30 | Plus 500 ng of HsRad51 | 55/98,000 | 56 |
| A−AG30 | Plus 1 μg of HsRad51 | 79/105,000 | 75 |
| A−AG30 | Plus 2 μg of HsRad51 | 103/97,000 | 106 |
| A+AG30 | No extract | 0/93,000 | 0 |
| A+AG30 | Standard | 39/105,000 | 37 |
| A+AG30 | Plus 500 ng of HsRad51 | 37/110,000 | 34 |
| A+AG30 | Plus 1 μg of HsRad51 | 54/114,000 | 47 |
| A+AG30 | Plus 2 μg of HsRad51 | 76/102,000 | 75 |

were seen. Hence, even though HsRad51, by itself, cannot fully complement the activity of the immunodepleted extracts, it can provide increased activity to otherwise complete whole cell extracts.

*The Role of the Nucleotide Excision Repair Factor, XPA*—In previous work studying TFO-induced mutagenesis and recombination within SV40 vectors in human cells, we had obtained genetic evidence that the ability of triplex formation to stimulate DNA metabolism is dependent on the activity of the NER pathway (8, 10). To obtain direct biochemical evidence in support of this proposed mechanism, we tested the requirement for the NER damage recognition factor, XPA (26), in the triplex-induced recombination in the HeLa cell extracts.

A rabbit polyclonal antibody was raised against recombinant human XPA protein produced in *E. coli* and was found to recognize a single protein in human cells of the expected size (data not shown). Using this antibody, XPA was removed from the extracts by immunoprecipitation. Depletion of XPA was confirmed by Western blot analysis of the residual samples (Fig. 4). Depletion of XPA from the extracts was found to substantially reduce the frequency of TFO-induced recombination, whether or not the TFO was covalently linked to the donor fragment (Table IV). With both the A−AG30 and the A+AG30 samples, the depletion of XPA reduced the frequency of recombinants to that mediated by the donor fragment alone (Table I). Hence, the ability of a triple helix to stimulate recombination depends on the XPA protein. This result supports the hypothesis that the NER pathway can recognize a triple helix as a "lesion," thereby provoking DNA metabolism that can lead to recombination or mutation.

Following XPA immunodepletion, we tested the ability of XPA to restore the triplex-induced recombination activity (Table IV). The results show that increasing amounts of XPA protein provide functional complementation in the depleted extracts. These results establish a direct role for XPA in mediating the ability of a triple helix to stimulate recombination.

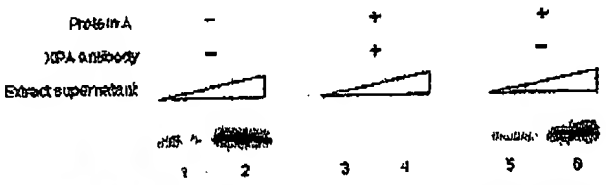



FIG. 4. **Immunodepletion of XPA protein from the HeLa cell extracts.** Extract samples were immunodepleted using a polyclonal XPA antibody pre-mixed with Protein A-Sepharose beads. The immunoprecipitate was removed by centrifugation, and the remaining supernatant was examined by Western blot analysis. Samples were treated as indicated.

## DISCUSSION

The work reported here establishes that triplex-induced recombination can be detected in human cell-free extracts. A 30-mer TFO that binds with high affinity to a portion of the *supFG1* reporter gene within the pSupFG1/G144C vector was found to stimulate recombination between the vector and a 40-nt donor fragment. Recombination was induced both when the donor fragment was linked to the TFO and when it was present as a separate, unlinked molecule. The stimulation was determined to occur in the extracts and not in the indicator bacteria because no recombinants were observed unless the samples were incubated in the extracts. The donor fragment, by itself, was able to participate in recombination with the plasmid in the extracts, consistent with previous studies that have detected intermolecular recombination in similar mammalian cell extracts (37). However, the present work establishes that such intermolecular recombination can be stimulated by third strand binding to one of the molecules.

Establishment of TFO-induced recombination allowed testing of the role of selected factors in the process. Immunodepletion of HsRad51, a human recombinase homologous to recA (34), from the extract reduced the yield of induced recombinants, but purified HsRad51 did not fully compensate for the immunodepletion. When the immunoprecipitate was re-solubilized and used to supplement the depleted extracts, the induced recombination activity was restored. These results suggest that the immunoprecipitate contains factors in addition to HsRad51 that are essential for the reaction. Such factors could include HsRad51-associated proteins that are proposed to play a role in homologous recombination, such as HsRad51a, HsRad51b, HsRad52, HsRad54, XRCC2, and XRCC3 (35, 36). On the other hand, the addition of extra HsRad51 to the non-depleted extracts produced an increased frequency of recombinants, suggesting that HsRad51, itself, plays a critical role in the process. This result is consistent with the observation that overexpressed HsRad51 can provide a modest increase in the frequency of recombination in reporter gene substrates in mammalian cells (38).

The NER damage recognition factor, XPA, was also found to play an essential role in the triplex-induced recombination, as no induced recombinants were seen in the extracts after XPA immunodepletion. Supplementation of the induced extracts with recombinant XPA protein restored the induced recombination activity. This result not only is consistent with previous work showing that triplex-induced mutagenesis and recombination are substantially reduced in human mutant cell lines deficient in XPA (8, 10), it also demonstrates directly that XPA is required for the process. Taken together, the data support a model in which the oligonucleotide-mediated triple helix is recognized by XPA, thereby initiating repair activity that can create recombinogenic intermediates. Such intermediates may

TABLE IV

*Effect of XPA depletion on triplex-induced recombination in cell-free extracts*

The results represent the combined data from three independent experiments.

| Oligonucleotide | Extract treatment | Blue colonies/total | Reversion frequency |
|---|---|---|---|
| | | | $\times 10^{-5}$ |
| None | Standard | 0/209,000 | 0 |
| A−AG30 | No extract | 0/213,000 | 0 |
| A−AG30 | Standard | 96/219,000 | 44 |
| A−AG30 | Protein A-Sepharose | 45/114,000 | 39 |
| A−AG30 | XPA antibody | 18/214,000 | 8 |
| A−AG30 | XPA antibody + 75 ng of XPA | 27/112,000 | 24 |
| A−AG30 | XPA antibody + 150 ng of XPA | 42/114,000 | 37 |
| A−AG30 | XPA antibody + 300 ng of XPA | 45/118,000 | 38 |
| A+AG30 | Standard | 46/118,000 | 39 |
| A+AG30 | Protein A-Sepharose | 39/111,000 | 35 |
| A+AG30 | XPA antibody | 12/115,000 | 10 |

either be correctly repaired, repaired with incorporation of mutation in an error-prone manner, or, if homologous DNA is present, serve as substrates for repair by a HsRad51-dependent pathway of homologous recombination.

In the extracts, the TFO was found to sensitize the plasmid to recombination either with a linked donor fragment or with an unlinked fragment, at approximately the same frequency. This ability of the TFO to stimulate recombination between the target site and an unlinked fragment is in contrast to a previous study examining TFO-induced recombination in COS cells, in which the linked TD-TFO molecule was found to be 4-fold more active than the mixture of the unlinked molecules (23). In that study, the pSupFG1/G144C vector was pre-transfected into COS cells, and the cells were transfected the next day with the oligonucleotides. Two days later, the vector DNA was isolated for analysis in indicator bacteria. We interpret this difference between the previous cell study and the present work to suggest that, in the *in vitro* reactions, the TFO and donor fragment are present in adequate local concentrations whether or not they are linked together covalently. Hence, in the extracts, the ability of the TFO to deliver a linked donor fragment to the target site and place it in juxtaposition with the region of homology is not as important in promoting recombination as is the ability of the triplex to provoke DNA metabolism. In contrast, in the cell experiments, both properties of the TFO appear to be needed, although it remains to be determined whether the need for linkage of the donor to the TFO in cells can be overcome by increasing the efficiency of donor fragment transfection. If so, it would allow a gene correction strategy in which a TFO could be used in combination with larger donor fragments, greater than those that can be synthesized in continuity with the TFO. This would be advantageous, since previous studies examining recombination between episomal or chromosomal targets and transfected DNAs in mammalian cells have consistently shown that fragments in the range of 500 bp or larger produce higher levels of recombination than do short fragments in the size range used here (31–33, 39, 40).

Overall, the work reported here demonstrates that triplex-induced recombination can be detected in human cell free extracts, and it provides insight into the underlying mechanism by identifying critical roles for HsRad51 and XPA. The results suggest a pathway of triplex-induced recombination that depends on NER and on homologous recombinational repair of NER-generated intermediates. The ability to reconstitute TFO-induced recombination *in vitro* should serve as a basis for further elucidation of the manner in which triplex formation can provoke DNA metabolism, and may thereby guide refinements in strategies to use TFOs to promote targeted genetic changes in human cells.

*Acknowledgments*—We thank Efim I. Golub and Charles M. Radding for generously providing help and reagents, Scott Wanager from the National Cell Culture Center for providing HeLa cells, and Lisa Cabral for help in preparing this manuscript. We also thank R. Franklin and S. Baserga for their help.

REFERENCES

1. Hanson, K. D., and Sedivy, J. M. (1995) *Mol. Cell. Biol.* 15, 45–61
2. Capecchi, M. R. (1989) *Science* 244, 1288–1292
3. Rouet, P., Smih, F., and Jasin, M. (1994) *Mol. Cell. Biol.* 14, 8096–8106
4. Rouet, P., Smih, F., and Jasin, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* 91, 6064–6068
5. Choulika, A., Perrin, A., Dujon, B., and Nicolas, J. F. (1995) *Mol. Cell. Biol.* 15, 1968–1973
6. Segal, D. J., and Carroll, D. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 806–810
7. Brenneman, M., Gimble, F. S., and Wilson, J. H. (1996) *Proc. Natl. Acad. Sci. U. S. A.* 93, 3608–3612
8. Wang, G., Seidman, M. M., and Glazer, P. M. (1996) *Science* 271, 802–805
9. Faruqi, A. F., Seidman, M. M., Segal, D. J., Carroll, D., and Glazer, P. M. (1996) *Mol. Cell. Biol.* 16, 6820–6828
10. Faruqi, A. F., Datta, H. J., Carroll, D., Seidman, M. M., and Glazer, P. M. (2000) *Mol. Cell. Biol.* 20, 990–1000
11. Luo, Z., Marrie, M. A., Faruqi, A. F., and Glazer, P. M. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 9003–9008
12. Vasquez, K. M., and Wilson, J. H. (1998) *Trends. Biochem. Sci.* 23, 4–9
13. Chan, P. P., and Glazer, P. M. (1997) *J. Mol. Med.* 75, 267–82
14. Praseuth, D., Guieysse, A. L., and Helene, C. (1999) *Biochim. Biophys. Acta* 1489, 181–206
15. Faria, M., Wood, C. D., Perrouault, L., Nelson, J. S., Winter, A., White, M. R., Helene, C., and Giovannangeli, C. (2000) *Proc. Natl. Acad. Sci. U. S. A.* 97, 3852–3857
16. Strobel, S. A., Doucette-Stamm, L. A., Riba, L., Housman, D. E., and Dervan, P. B. (1991) *Science* 254, 1639–1642
17. Gunther, E. J., Havre, P. A., Gasparro, F. P., and Glazer, P. M. (1998) *Photochem. Photobiol.* 68, 207–212
18. Giovannangeli, C., Divinacco, S., Labrousse, V., Gryaznov, S., Chernezu, P., and Helene, C. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 79–84
19. Wang, G., Levy, D. D., Seidman, M. M., and Glazer, P. M. (1995) *Mol. Cell. Biol.* 15, 1759–1768
20. Majumdar, A., Khorlin, A., Dyatkina, N., Lin, F.-L. M., Powell, J., Liu, J., Fei, Z., Khripine, Y., Watanabe, K. A., George, J., Glazer, P. M., and Seidman, M. M. (1998) *Nat. Genet.* 20, 212–214
21. Oh, D. H., and Hanawalt, P. C. (1999) *Nucleic Acids Res.* 27, 4734–4742
22. Belousov, E. S., Afonina, I. A., Kutyavin, I. V., Gall, A. A., Reed, M. W., Gamper, H. B., Wydro, R. M., and Meyer, R. B. (1998) *Nucleic Acids Res.* 26, 1324–1328
23. Chan, P. P., Lin, M., Faruqi, A. F., Powell, J., Seidman, M. M., and Glazer, P. M. (1999) *J. Biol. Chem.* 274, 11541–11548
24. Gamper, H. B., Hou, Y. M., Shamm, M. R., Podymnogin, M. A., and Meyer, R. B. (1998) *J. Am. Chem. Soc.* 120, 2182–2183
25. Broitman, S., Amosova, O., Dolinnaya, N. G., and Fresco, J. R. (1999) *J. Biol. Chem.* 274, 21769–21768
26. Sancar, A. (1996) *Annu. Rev. Biochem.* 65, 43–81
27. Gupta, R. C., Bazemore, L. R., Golub, E. I., and Radding, C. M. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 463–468
28. Glazer, P. M., Sarkar, S. N., Chisholm, G. E., and Summers, W. C. (1987) *Mol. Cell. Biol.* 7, 218–224
29. Hanf, T., Golub, E. I., Reddy, G., Radding, C. M., and Ward, D. C. (1995) *Proc. Natl. Acad. Sci. U. S. A.* 92, 2298–2302
30. Jones, C. J., and Wood, R. D. (1993) *Biochemistry* 32, 12096–12104
31. Campbell, C. R., Keown, W., Lowe. L., Kirschling, D., and Kucherlapati, R. (1989) *New Biol.* 1, 223–227
32. Gonzo, K. K., and Gruenert, D. C. (2000) *Methods Mol. Biol.* 133, 85–99
33. Lin, F. L., Sperle, K. M., and Sternberg, N. L. (1987) *Mol. Cell. Biol.* 7, 129–140
34. Baumann, P., and West, S. C. (1998) *Trends Biochem. Sci.* 23, 247–251
35. Thacker, J. (1999) *Trends Genet.* 15, 166–168
36. Sung, P., Trujillo, K. M., and Van Komen, S. (2000) *Mutat. Res.* 451, 257–275
37. Kucherlapati, R. S., Ayares, D., Hannoken, A., Noonan, K., Rauth, S., Spencer, J. M., Wallace, L., and Moore, P. D. (1984) *Cold Spring Harbor Symp. Quant. Biol.* 49, 191–197
38. Yanez, R. J., and Porter, A. C. (1999) *Gene Ther.* 6, 1282–1290
39. Donoho, G., Jasin, M., and Berg, P. (1998) *Mol. Cell. Biol.* 18, 4070–4078
40. Taghian, D. G., and Nickoloff, J. A. (1997) *Mol. Cell. Biol.* 17, 6386–6393

PERSPECTIVE SERIES
Genetic repair | Bruce A. Sullenger, Series Editor

# The potential for gene repair via triple helix formation

Michael M. Seidman[1] and Peter M. Glazer[2]

[1]National Institute on Aging, NIH, Baltimore, Maryland, USA
[2]Department of Therapeutic Radiology and Department of Genetics, Yale University School of Medicine, New Haven, Connecticut, USA

Triplex-forming oligonucleotides (TFOs) can bind to polypurine/polypyrimidine regions in DNA in a sequence-specific manner. The specificity of this binding raises the possibility of using triplex formation for directed genome modification, with the ultimate goal of repairing genetic defects in human cells. Several studies have demonstrated that treatment of mammalian cells with TFOs can provoke DNA repair and recombination, in a manner that can be exploited to introduce desired sequence changes. This review will summarize recent advances in this field while also highlighting major obstacles that remain to be overcome before the application of triplex technology to therapeutic gene repair can be achieved.

*J. Clin. Invest.* 112:487–494 (2003). doi:10.1172/JCI200319552.

## Triple-helix DNA

DNA triple helices form in a sequence-specific manner on polypurine:polypyrimidine tracts (1–3) which are widespread in mammalian genomes (4–6). The third strand lies in the major groove of an intact duplex (Figure 1) and is stabilized by two Hoogsteen hydrogen bonds between third strand bases and the purines in the duplex (3, 7). The third strand may consist of pyrimidines, or purines, depending on the nature of the target sequences (Figures 2 and 3). In the pyrimidine (or Y.R:Y) motif, a homopyrimidine oligonucleotide binds in a direction parallel to the purine strand in the duplex, with canonical base triplets of T.A:T and C.G:C. In the alternate purine motif (R.R:Y), a homopurine strand binds antiparallel to the purine strand, with base triplets of A.A:T and G.G:C (8, 9).

The demonstration that synthetic oligonucleotides could form stable triplexes (10, 11) suggested that TFOs could be developed as sequence-specific gene targeting reagents in living cells (12–17). However, a number of obstacles have been, and still must be, overcome. Triplex chemistry and biochemistry impose fundamental limitations to TFO activity in the nuclear environment, and target options are limited to polypurine:polypyrimidine sequences. In addition, it has been shown that nucleosomes can inhibit triplex formation (18–20). Consequently, accessibility of genomic targets is an important issue.

Recent developments in nucleoside and oligonucleotide analogue chemistry show great promise for solving problems of TFO bioactivity and target options. We will discuss the challenges posed by the cellular environment and target restrictions, and some of the chemistry that may address these issues. We will also consider the activity of TFOs in biological assays. We will not attempt an in depth review of the chemistry of TFOs. Instead, we will stress some of the strategic themes and cite a few specific examples. The interested reader should see (21–23) for excellent and comprehensive discussions of TFO chemistry.

## Obstacles to TFO activity under physiological conditions

Biological applications of TFOs are compromised by fundamental biophysical considerations, as well as limitations imposed by physiological conditions. Triplex formation involves the approach and binding of a negatively charged third strand to a double-negatively charged duplex. Neutralization of charge repulsion is typically provided experimentally by levels of $Mg^{++}$ (5–10 mM) (24) that are much higher than what is thought to be available in cells (25). Furthermore, triplex formation involves conformational changes on the part of the third strand, and some distortion of the underlying duplex (26–28). Pyrimidine motif triplexes are unstable at physiological pH

Address correspondence to: Peter M. Glazer, Department of Therapeutic Radiology, Yale University School of Medicine, P.O. Box 208040, New Haven, Connecticut 06520-8040, USA. Phone: (203) 737-2788; Fax: (203) 737-2630; E-mail: peter.glazer@yale.edu.

Conflict of interest: The authors have declared that no conflict of interest exists.

Nonstandard abbreviations used: triplex-forming oligonucleotide (TFO); 2-aminopyridine (2AP); 2′-*O*-methoxy (OMe); 2-aminoethyl (AE); equilibrium dissociation constant (Kd); hypoxanthine phosphoribosyltransferase (HPRT); *Xeroderma pigmentosum* group A protein (XPA); thymidine kinase (TK).

because of the requirement for cytosine protonation that occurs at relatively acidic pH (pKa = 4.5). This is necessary for the second Hoogsteen hydrogen bond, although the resultant positive charge apparently makes the more important contribution to triplex stability (29). Pyrimidine motif triplexes containing adjacent cytosines are often less stable than those with isolated cytosines. Traditionally this has been ascribed to charge-charge repulsion effects (30), although a recent study suggests incomplete protonation of adjacent cytosines may be the critical factor (31). In addition, purine motif third strands (which are G rich) may form G tetrads in physiological levels of $K^+$, which inhibit triplex formation (32). All these factors impose kinetic barriers on triplex formation and reduce the stability of triplexes once formed (most triplexes, even under optimal conditions in vitro, are less stable than the underlying duplex).

### Oligonucleotide modifications improve TFO activity under physiological conditions

The use of 5-methylcytosine partially alleviates the pH restriction of TFOs in the pyrimidine motif (33, 34). This is thought to be due to the contribution of the methyl group in the major groove to base stacking (35), and/or the exclusion of water molecules from the groove (36). Cytosine has also been replaced with analogues such as 8-oxoadenine (37), pseudoisocytidine (38), and a 6-keto derivative of 5-methylcytydine (39). These can form two hydrogen bonds at physiological pH, and may be useful for sequence targets with adjacent cytosines. Two groups have presented interesting data regarding 2-aminopyridine (2AP) (40, 41). This analogue is protonated at neutral pH and stable triplexes were formed by TFOs containing adjacent 2AP residues on targets with adjacent cytosines. These results are consistent with the argument that proton competition between adjacent cytosines is the basis for the difficulty of targets with adjacent cytosines (31). Although the biochemical data with these analogues are encouraging, only 5-methylcytosine has been tested in biological assays.

RNA third strands formed more stable pyrimidine-motif triplexes than the corresponding DNA strands (42), which prompted the use in third strands of 2′-O-Methoxy (OMe) sugar residues (27, 43), and recently, 2′, 4′ bridged ribose substitutions (44). These modifications preorganize the third strand in a conformation that is compatible with triplex formation and imposes minimal distortion on the underlying duplex (28, 44). Other modifications also improve stability. Intercalators linked to TFOs improve binding (45), propynyl-deoxyuridine reduces the $Mg^{++}$ dependence (46), as does replacement of the ribose with a morpholino analogue (47).

Backbone modifications that replace the phosphate linkage (48), or a bridging oxygen atom with a nitrogen (49) improve TFO binding in vitro. Replacement of a nonbridging oxygen atom in the backbone with a charged amine reduces the likelihood of self-structure formation of purine TFOs in physiological $K^+$ (50, 51). A positive charge on a thymidine analogue (52), or linkage of positively charged moieties to TFOs, also enhances triplex stability (53, 54).

A positive charge and an RNA-like sugar conformation have been joined in the 2′-O-(2-aminoethyl) (AE) ribose derivatives developed by Cuenoud and colleagues (55–57). TFOs carrying these substitutions show enhanced kinetics of triplex formation and greater stability of the resultant complex at physiological pH and low $Mg^{++}$ concentration. NMR analysis indicates a specific interaction between the positively charged amines (at physiological pH) and phosphate groups in the purine strand of the duplex (58). A related approach has been described in a thymidine analogue containing both 5-aminopropyl-largyl and 2′-aminoethoxy moieties (59).

### Biological activity of TFOs

In early biological studies, TFOs were envisioned as tools to inhibit gene expression by blocking transcription initiation or elongation (the "antigene" strategy) (60). Triplex formation within promoter sites has been shown to block transcription factor access and inhibit gene activation in vitro, and several studies have demonstrated that TFOs can decrease gene expression in mammalian cells in a directed way (see ref. 61 for a recent description of this approach). As an alternative strategy for genetic manipulation, we, and others, have investigated the use of TFOs to mediate genome modification, resulting in a change in target sequence (13, 17, 62–70). This has the advantage of introducing permanent changes in the target sequence, which simplifies interpretation of the experiments. However, more importantly, it also has potential as a gene knockout tool and as a means for gene correction.

In our initial work, the third strand was conjugated to a mutagen, such as psoralen, so that the sequence specificity of the third strand binding could be conferred on the action of the mutagen (71, 72). Psoralen-conjugated TFOs transfected into monkey COS cells could induce base pair–specific mutations within a *supF* mutation reporter gene in a simian virus 40-based plasmid episome in the cells, at frequencies in the range of 1–5% (16). The key finding in this work was that the binding affinity of the TFO to its target site, as measured in vitro, was highly correlated with its intracellular activity. TFOs with equilibrium dissociation constants (Kd's) of approximately $10^{-9}$ M were active; those with Kd's of $10^{-6}$ M were not.

This work was subsequently extended to psoralen-TFO–mediated knockout of chromosomal genes. In one study, the supFG1 reporter integrated into the chromosome of mouse fibroblasts was used as a target (73). Again, only high-affinity TFOs were active, achieving targeted mutagenesis frequencies of 0.1%. In the supFG1 experiments, essentially unmodified G-rich oligonucleotides (except for 3′ end capping) designed

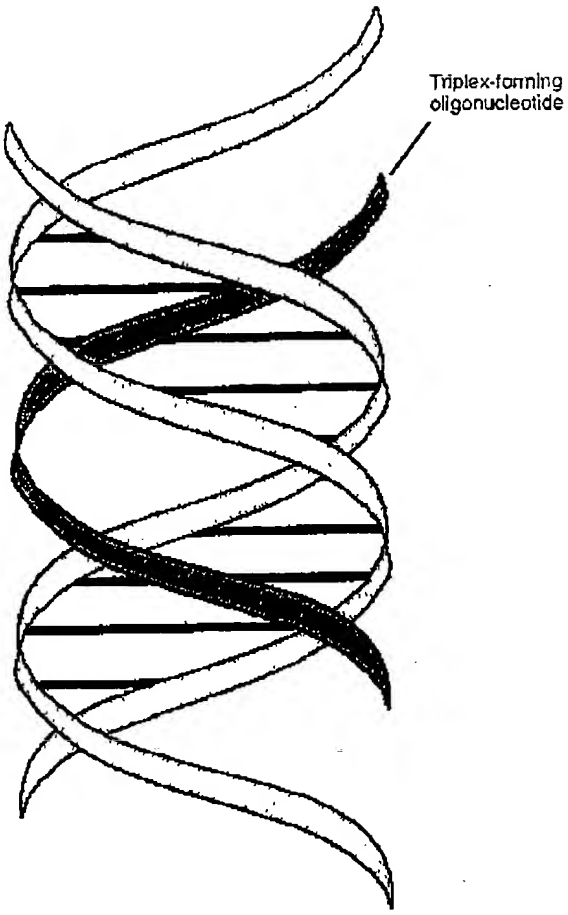



**Figure 1**
Diagrammatic depiction of a DNA triple helix, with the third strand binding in the major groove.

to bind in the antiparallel purine motif were used, but it should be noted that in those experiments the 30-bp polypurine site in the *supFG1* gene afforded the possibility of high affinity third-strand binding by G-rich TFOs synthesized with standard DNA chemistry (16).

We have determined the activity of variously modified pyrimidine motif psoralen-TFOs, designed to target a sequence in the endogenous hamster hypoxanthine phosphoribosyltransferase (HPRT) gene, in HPRT knockout assays (17). TFOs with uniform 2′-*O*-methyl substitutions were inactive, but addition of a pyrene intercalator conferred knockout activity with *HPRT*⁻ clones recovered at frequencies in the range of $10^{-4}$ to $10^{-3}$. Sequence analysis of the mutant clones confirmed the localization of mutations to the target region (17). More recently, we have prepared psoralen-TFOs containing several AE residues as well as 2′-*O*-methyl substitutions. These mixed substitution TFOs form triplexes at lower levels of $Mg^{++}$ than required for the TFOs with only the 2′-*O*-methyl residues. Thermal melting experiments showed that the AE triplexes were more stable than the 2′-*O*-methyl only triplexes, and, notably, were more stable than the underlying duplex at physiological pH. They were also more stable in the cellular compartment in which replication and mutagenesis occur (62, 74). Most importantly they are quite active in the *HPRT* knockout assay (62). These results indicate that the introduction of positive charge can confer biological activity on pyrimidine motif TFOs.

Other oligonucleotide modifications also support biological activity. For example, a psoralen-linked pyrimidine TFO with a phosphoramidate backbone (in this case, substitution of a bridging oxygen with nitrogen) was shown to have statistically significant activity in a yeast reversion assay (75). Psoralen-linked G-rich TFOs in the purine motif, with a nonbridging phosphoramidate substitution (N,N-diethyl-ethylenediamine), were active in targeted mutagenesis experiments at a short (10 bp) homopurine/homopyrimidine site in mammalian cells that otherwise is not susceptible to targeting by unmodified phosphodiester oligonucleotides (67). Improved binding activity in reduced $Mg^{++}$ is probably the basis for the bioactivity of the TFOs carrying these modifications.

In addition to the use of psoralen-conjugated TFOs in site-directed mutagenesis studies in mammalian cells and in yeast, a few groups have used these reagents as tools for direct physical demonstrations of triplex formation in permeabilized mammalian cells. Such studies have used PCR and restriction enzyme protection assays to argue for the formation of TFO-targeted crosslinks in genomic DNA in chromatin (76–78). These results, and the mutagenesis experiments, show that the packaging of the DNA into chromatin is not an absolute barrier to gene targeting with TFOs.

### Triplex formation by itself can promote DNA metabolism

In the course of our work with psoralen-TFOs, we observed that unconjugated TFOs were also capable of inducing mutations in the target gene in an SV40 vector, at least when the binding affinity was sufficiently high (79). This effect was shown to be a consequence of the stimulation of DNA repair by the formation of the triple helix, which seems to be recognized by the nucleotide excision repair complex as a lesion.

Recently, Vasquez et al. demonstrated that systemically administered TFOs (without psoralen or any other DNA reactive conjugate) could induce mutations at specific genomic sites in the somatic cells of adult mice (68). In this work, a 30-mer purine TFO, with a 3′ propanolamine group to prevent exonuclease-mediated degradation, was injected intraperitoneally for 5 consecutive days. The TFO was targeted to chromosomal copies of an integrated *supF* reporter gene. After an additional 10 days, the mice were sacrificed, and tissues were taken for mutation

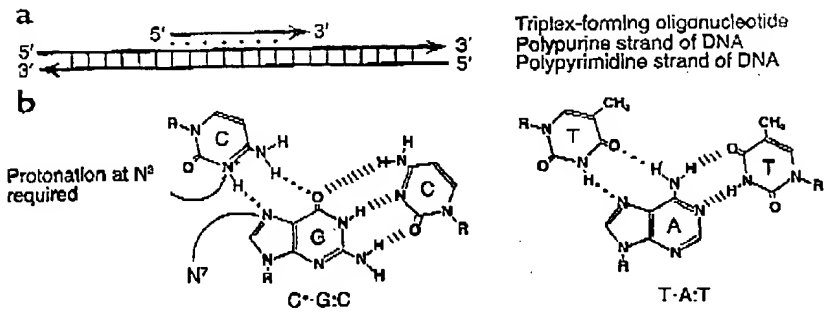



**Figure 2**
Diagram of the pyrimidine motif for triple helix formation. (a) Orientation of the third strand in the pyrimidine triple-helix motif. Note that the third strand is parallel in terms of 5' to 3' orientation with respect to the purine-rich strand of the duplex target. (b) Base triplets formed in the pyrimidine motif and illustration of the Hoogsteen hydrogen bonds that stabilize triple-helix formation.

analysis. In general, mice treated with the sequence-specific TFO had a fivefold elevated mutation rate in the targeted *supF* gene, but not in the nontargeted *cII* gene. All tissues tested showed TFO-induced mutagenesis except the brain, which had no mutagenesis over background, consistent with TFOs inability to cross the blood-brain barrier. This work established that site-directed DNA binding molecules, upon systemic administration, can mediate gene targeting and gene modification in vivo in whole animals.

### Triplex-induced recombination: gene correction via recombinational repair

Based on the concept that third-strand binding, with or without psoralen coupling, can trigger DNA repair, we hypothesized that such binding might also be recombinogenic due to the production of repair-dependent DNA strand breaks. Using an SV40 vector containing two mutant copies of the *supF* gene, we found that both psoralen-TFOs (64) and non-psoralen TFOs (63) could trigger recombination within an SV40 virus genome. This induced recombination, in the case of the TFOs, without psoralen, was found to be dependent on the presence of functional *Xeroderma pigmentosum* group A protein (XPA) protein (63), the key recognition factor in nucleotide excision repair, a result consistent with our hypothesis.

These results were extended to a chromosomal target, in which two mutant thymidine kinase (TK) genes were integrated into a single chromosomal site in mouse fibroblasts (69). Transfection (via cationic liposomes) of the cells with high-affinity TFOs targeting a region between the two TK genes yielded recombination at a frequency of approximately $30 \times 10^{-6}$, about sevenfold above background. When the TFOs were microinjected into the nuclei of the cells (about 70,000 copies/cell), the yield of recombinants increased to 1–2%, more than 1000-fold over background. Analysis of the recombinant clones revealed all the recombination events involved gene conversion rather than cross-over recombination. Similar work to use TFOs to stimulate recombination has been described by Wilson and colleagues using a locus in CHO cells containing duplicated *APRT* genes as a target (80). Besides providing proof-of-principle, the results obtained in the TK system highlight another challenge in using triplex technology for gene repair: achieving efficient delivery into the cell nucleus.

### Bifunctional oligonucleotides for gene correction

The observation of the ability of third strand binding to provoke DNA repair and stimulate recombination led us to develop a strategy to mediate targeted gene conversion using a TFO linked to a short DNA fragment homologous to the target site (except for the base pair to be corrected) (70). In this bifunctional molecule, the TFO domain mediates site-specific binding to target the molecule to the desired gene. This binding also triggers repair to sensitize the target site to recombination. The tethered homologous donor fragment can participate in recombination and/or gene conversion with the target gene to correct or alter the nucleotide sequence.

Using a bifunctional oligomer with a 40-mer donor domain and a 30-mer TFO domain, correction of a single base-pair mutation in the *supF* reporter gene within an SV40 vector in COS cells was achieved (70). Correction frequencies were in the range of 0.1–0.5% with the full bifunctional molecule. Oligomers consisting of either domain above or of either domain substituted with heterologous sequences reduced activity by tenfold or more. The donor domain alone consistently did mediate some gene correction, as would be expected, based on the known ability of short DNA fragments to mediate some level of recombination (81–83). However, there was a clear synergism due to combination with the TFO domain.

In vitro studies in human cell-free extracts further demonstrated that triplex formation could induce recombination of the target site with a short donor oligonucleotide, and they also revealed a requirement for XPA and Rad51 function in the pathway (84). Importantly, the in vitro work also revealed that the donor DNA does not need to be covalently linked to the TFO, suggesting that the ability of the third strand to stimulate repair and recombination is a key property, presumably because of the production of strand breaks as recombinogenic intermediates.

Several other in vitro studies have demonstrated synergistic binding of bifunctional oligonucleotides containing domains designed to form triplexes as well

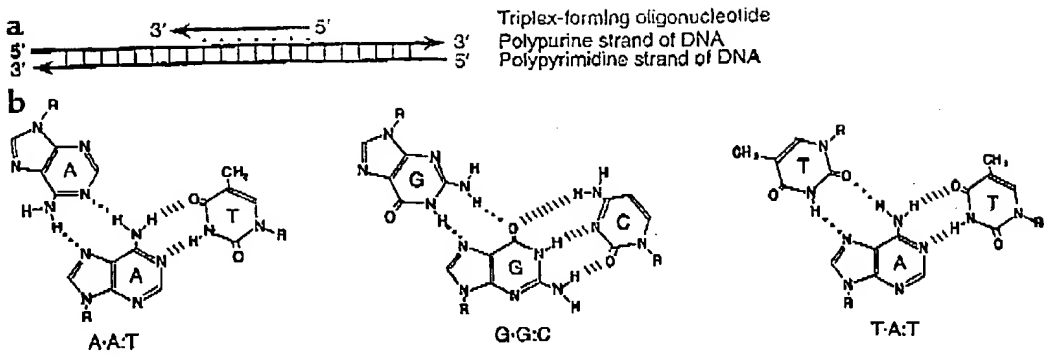



**Figure 3**
Diagram of the purine motif for triple-helix formation. (a) Orientation of the third strand in the purine triple-helix motif. Note that the third strand is oriented antiparallel in terms of 5' to 3' direction with respect to the purine-rich strand of the duplex target. (b) Base triplets formed in the purine motif and illustration of the reverse Hoogsteen hydrogen bonds that stabilize triple-helix formation.

as duplexes with a target DNA (66, 85, 86). Interestingly, in one strategy, the triplex-forming domain was linked to the duplex-forming domain by annealing via a short stretch of complementary nucleotides (86), rather than via covalent linkage (the latter either by postsynthetic coupling or by cosynthesis as a single, long oligonucleotide).

### Prospects for the expansion of the triplex-binding code

Although we have just begun to explore the applications of TFOs for genome modification, we would argue that two fundamental issues have been resolved in recent years: that it is possible to prepare TFOs with gene-targeting activity in vivo, and that bioactivity implies at least some degree of target accessibility. Thus, although there is a great deal to be done to extend these conclusions, expansion of target options to mixed purine/pyrimidine sequences would appear to be the most important challenge facing the field. We will discuss some of the efforts to expand the triplex-binding code, although it should be pointed out that only a few compounds have been tested in bioassays. For thorough accounts of this aspect of the field the reader should consult recent reviews (21, 23).

Purine bases engaged in Watson Crick pairing can form two additional hydrogen bonds, while duplex pyrimidines can only form one additional hydrogen bond. These hydrogen bonding patterns are the basis for much of the stability of conventional triplexes, and one of the reasons for the instability of triplexes on mixed sequences. An additional problem presented by T:A inversions is the projection into the major groove by the 5-methyl group of T resulting in steric hindrance to groove occupancy by third strands. Candidate compounds for binding at inversion sites fall into different categories: natural bases, intercalators, analogues that make a single hydrogen bond with the inverted pyrimidine base, analogues that make hydrogen bonds with the purine base, and, perhaps, also with the pyrimidine base (base pair binders).

Natural bases in parallel triplexes have been shown to form triplets at inverted base pairs, specifically G.T:A, and T.C:G (87, 88). Although these have been incorporated in TFOs employed in biological experiments, triplexes containing them are not as stable as perfectly matched triplexes (89, 90). Thus we found that a TFO containing G, for triplet formation at a T:A inversion site, was not effective in our *hprt* gene knockout assay (17). The T.C:G triplet can form in both parallel and antiparallel triplexes. Use in parallel triplexes is compromised by the lack of sequence stringency (since T also forms triplets with A:T pairs). However this limitation does not apply to antiparallel triplexes and we have used T in a biologically active purine motif TFO (AG30, described above) for triplet formation at T:A sites in an otherwise perfect polypurine target (16).

Intercalators, incorporated into the TFOs and located adjacent to mismatch sites, have been used to stabilize natural base triplets with inverted base pairs. An acridine derivative stabilized either inversion depending on its position relative to the site (45). A naphthalene-based intercalator linked to 5-methylcytosine, designed to form one hydrogen bond and also intercalate, showed selectivity for C:G inversions (91). We introduced a pyrene derivative at a T:A inversion site in our active TFO in our initial demonstration of *HPRT* gene knockout (17). It is clear that intercalators can enhance triplex stability, however it is likely that this will be at the cost of some degree of sequence specificity. Precisely this observation was made with one of the earliest efforts to construct an analogue for expansion of target options. A benzamidophenyl derivative of imidazole formed stable triplets at both T:A and C:G base pairs by intercalation next to the target base pairs (92, 93).

There have been a number of attempts to synthesize compounds with hydrogen bonding potential that would form triplets with inverted pairs with good affinity and specificity. There have been some promising developments with the C:G inversion,

particularly in the pyrimidine motif. A thymidine analogue designed to form a hydrogen bond with the C showed selectivity relative to the other base pairs (94). Similar results with a related compound have been reported in one of the few instances in which the new sugar chemistry has been combined with a base analogue (95). A base pair recognition strategy was pursued by Miller and colleagues who prepared several $N^4$-substituted cytosine derivatives with side-chain extensions designed to make hydrogen bonds with both the cytosine and the guanine in the inverted base pair (96, 97). This approach is conceptually attractive because formation of hydrogen bonds with both bases should enhance the specificity of the interaction with the intended base pair, as indeed was observed with some of the derivatives (97). While these and other analogues show promise for solving the C:G inversion they do not form triplets as strong as the canonical C+.G:C. However, this is, in part, because they do not carry a positive charge. Combination with sugar and backbone modifications that introduce positive charge may overcome this limitation.

Analogues that bind C:G interruptions in antiparallel purine-motif triplexes have also been described. An example of an analogue that can reach across the major groove and form hydrogen bonds with the guanine base in the inverted C:G pair is 2′-deoxyformycin A (98). Another interesting approach involved the replacement of natural bases with azole derivatives. The use of the smaller aromatic ring was intended to overcome some of the steric problems associated with base pair inversions (99). They were able to demonstrate enhanced binding by the substituted TFOs to targets containing inversions, but base pair discrimination was poor. However, this may be a useful point of departure for side-chain derivatives that would improve selectivity.

Current efforts to overcome the T:A inversion have been less successful. A recent effort employed a post synthetic modification strategy, at an internal acyclic linker, to prepare a number of candidates (100). Although this approach has a great deal of potential, the most effective binders recovered in the study were intercalators, which failed to distinguish between T:A and C:G base pairs in the target sequence. This result is similar to those reported previously and suggests that in further work analogues with intercalative potential should be avoided. Finally, a pyridazine derivative on an acyclic linker has been shown to form triplets with T:A pairs (101). This analogue showed reasonable base pair discrimination, but was analyzed in the context of a peptide nucleic acid, and has not been examined in an oligonucleotide.

While this is a cursory overview, it is striking how much thoughtful chemistry has been described, some with considerable promise, and how rare has been the analysis of these developments in biological assays.

## Summary and future directions

Overall, the work to date suggests that TFOs can be used to mediate site-specific genome modification. This capacity derives not only from the ability of TFOs to bind as third strands with sequence specificity but also from the ability of the resulting triple helices, or TFO-mutagen complexes, to provoke repair and recombination, leading to directed mutagenesis, recombination, and, potentially, gene correction. It seems likely that recent advances in oligonucleotide chemistry have considerable potential for the development of TFOs with robust gene targeting activity. This will require coordinated effort between the chemists and biologists, but recent data suggest that this effort will be rewarded.

*Note added in proof.* B.J. Gold and colleagues have recently described a novel approach towards the development of TFOs that can bind general sequences (102).

## Acknowledgments

This work was supported by the NIH (grants GM54731 and CA64186 to P.M. Glazer).

1. Felsenfeld, G., Davies, D.R., and Rich, A. 1957. Formation of a three stranded polynucleotide molecule. *J. Am. Chem. Soc.* 79:2023–2024.
2. Thuong, N.T., and Helene, C. 1993. Sequence specific recognition and modification of double helical DNA by oligonucleotides. *Angewandte Chemie, Int. Ed. Eng.* 32:666–690.
3. Frank-Kamenetskii, M.D., and Mirkin, S.M. 1995. Triplex DNA structures. *Annu. Rev. Biochem.* 64:65–95.
4. Manor, H., Rao, B.S., and Martin, R.G. 1988. Abundance and degree of dispersion of genomic d(GA)n.d(TC)n sequences. *J. Mol. Evol.* 27:96–101.
5. Schroth, G.P., and Ho, P.S. 1995. Occurrence of potential cruciform and H-DNA forming sequences in genomic DNA. *Nucleic Acids Res.* 23:1977–1983.
6. Behe, M.J. 1995. An overabundance of long oligopurine tracts occurs in the genome of simple and complex eukaryotes. *Nucleic Acids Res.* 23:689–695.
7. Radhakrishnan, I., and Patel, D.J. 1994. DNA triplexes: solution structures, hydration sites, energetics, interactions, and function. *Biochemistry.* 33:11405–11416.
8. Possella, J.A., Kim, Y.J., Shih, H., Richards, E.G., and Fresco, J.R. 1993. Relative specificities in binding of Watson-Crick base pairs by third strand residues in a DNA pyrimidine triplex motif. *Nucleic Acids Res.* 21:4511–4515.
9. Lenai, A.G., Palladino, M.A., Fromm, E., Rizzo, V., and Fresco, J.R. 1988. Specificity in formation of triple-stranded nucleic acid helical complexes: studies with agarose-linked polyribonucleotide affinity columns. *Biochemistry.* 27:9108–9112.
10. Moser, H.E., and Dervan, P.B. 1987. Sequence-specific cleavage of double helical DNA by triple helix formation. *Science.* 238:645–650.
11. Le Doan, T., et al. 1987. Sequence-specific recognition, photocrosslinking and cleavage of the DNA double helix by an oligo-[alpha]-thymidylate covalently linked to an azidoproflavine derivative. *Nucleic Acids Res.* 15:7749–7760.
12. Helene, C., Thuong, N.T., and Harel-Bellan, A. 1992. Control of gene expression by triple helix-forming oligonucleotides. The antigene strategy. *Ann. N. Y. Acad. Sci.* 660:27–36.
13. Vasquez, K.M., and Wilson, J.H. 1998. Triplex-directed modification of genes and gene activity. *Trends Biochem. Sci.* 23:4–9.
14. Maher, L.J., III. 1996. Prospects for the therapeutic use of antigene oligonucleotides. *Cancer Invest.* 14:66–82.
15. Neidle, S. 1997. Recent developments in triple-helix regulation of gene expression. *Anticancer Drug Des.* 12:433–442.
16. Wang, G., Levy, D.D., Seidman, M.M., and Glazer, P.M. 1995. Targeted mutagenesis in mammalian cells mediated by intracellular triple helix formation. *Mol. Cell Biol.* 15:1759–1768.
17. Majumdar, A., et al. 1998. Targeted gene knockout mediated by triple helix forming oligonucleotides. *Nat. Genet.* 20:212–214.
18. Brown, P.M., and Fox, K.R. 1996. Nucleosome core particles inhibit DNA triple helix formation. *Biochem. J.* 319:607–611.

19. Espinas, M.L., Jimenez-Garcia, E., Martinez-Balbas, A., and Azorin, F. 1996. Formation of triple-stranded DNA at d(GA.TC)n sequences prevents nucleosome assembly and is hindered by nucleosomes. *J. Biol. Chem.* 271:31807–31812.
20. Bailey, C.P., Dagle, J.M., and Weeks, D.L. 1998. Cationic oligonucleotides can mediate specific inhibition of gene expression in Xenopus oocytes. *Nucleic Acids Res.* 26:4860–4867.
21. Gowers, D.M., and Fox, K.R. 1999. Towards mixed sequence recognition by triple helix formation. *Nucleic Acids Res.* 27:1569–1577.
22. Fox, K.R. 2000. Targeting DNA with triplexes. *Curr. Med. Chem.* 7:17–37.
23. Luyten, I.A., and Herdewijn, P. 2002. Hybridization properties of base-modified oligonucleotides within the double and triple helix motif. *Eur. J. Med. Chem.* 33:515–576.
24. Blume, S.W., et al. 1999. The integral divalent cation within the intermolecular purine*purine-pyrimidine structure: a variable determinant of the potential for and characteristics of the triple helical association. *Nucleic Acids Res.* 27:695–702.
25. Pesco, J., Salmon, J.M., Vigo, J., and Viallet, P. 2001. Mag-indol affinity for Ca(2+), compartmentalization and binding to proteins: the challenge of measuring Mg(2+) concentrations in living cells. *Anal. Biochem.* 290:221–231.
26. Gilbert, D.E., and Feigon, J. 1999. Multistranded DNA structures. *Curr. Opin. Struct. Biol.* 9:305–314.
27. Shimizu, M., Konishi, A., Shimada, Y., Inoue, H., and Ohtsuka, E. 1992. Oligo(2'-O-methyl)ribonucleotides. Effective probes for duplex DNA. *FEBS Lett.* 302:155–158.
28. Asensio, J.L., Cuest, R., Brown, T., and Lane, A.N. 1999. Conformational and thermodynamic properties of parallel intramolecular triple helixes containing a DNA, RNA, or 2'-OMeDNA third strand. *J. Am. Chem. Soc.* 121:11063–11070.
29. Asensio, J.L., Lane, A.N., Dhesi, J., Bergqvist, S., and Brown, T. 1998. The contribution of cytosine protonation to the stability of parallel DNA triple helices. *J. Mol. Biol.* 275:811–822.
30. Volker, J., and Klump, H.H. 1994. Electrostatic effects in DNA triple helices. *Biochemistry.* 33:13502–13508.
31. Sugimoto, N., Wu, P., Hara, H., and Kawamoto, Y. 2001. pH and cation effects on the properties of parallel pyrimidine motif DNA triplexes. *Biochemistry.* 40:9396–9405.
32. Arimondo, P.B., Garestier, T., Helene, C., and Sun, J.S. 2001. Detection of competing DNA structures by thermal gradient gel electrophoresis: from self-association to triple helix formation by (G,A)-containing oligonucleotides. *Nucleic Acids Res.* 29:E15.
33. Lee, J.S., Woodsworth, M.L., Latimer, L.J., and Morgan, A.R. 1984. Poly(pyrimidine).poly(purine) synthetic DNAs containing 5-methylcytosine form stable triplexes at neutral pH. *Nucleic Acids Res.* 12:6603–6614.
34. Povsic, T.J., and Dervan, P.B. 1989. Triple helix formation by oligonucleotides on DNA extended to the physiological range. *J. Am. Chem. Soc.* 111:3059–3061.
35. Singleton, S.F., and Dervan, P.B. 1992. Influence of pH on the equilibrium association constants for oligodeoxyribonucleotide-directed triple helix formation at single DNA sites. *Biochemistry.* 31:10995–11003.
36. Xodo, L.E., Manzini, G., Quadrifoglio, F., van der Marel, G.A., and van Boom, J.H. 1991. Effect of 5-methylcytosine on the stability of triple-stranded DNA—a thermodynamic study. *Nucleic Acids Res.* 19:5625–5631.
37. Miller, P.S., Bi, G., Kipp, S.A., Fok, V., and DeLong, R.K. 1996. Triplex formation by a psoralen-conjugated oligodeoxyribonucleotide containing the base analog 8-oxo-adenine. *Nucleic Acids Res.* 24:730–736.
38. Ono, A., Ts'o, P.O., and Kan, L. 1991. Triple helix formation of oligonucleotides containing 2'-O-methylpseudoisocytidine in substitution for 2'-deoxycytidine. *J. Am. Chem. Soc.* 113:4032–4033.
39. Xiang, G.B., and McLaughlin, L.W. 1998. A cytosine analogue containing a conformationally flexible acyclic linker for triplex formation at sites with contiguous G-C base pairs. *Tetrahedron.* 54:375–392.
40. Cassidy, S.A., et al. 1997. Recognition of GC base pairs by triplex forming oligonucleotides containing nucleosides derived from 2-aminopyridine. *Nucleic Acids Res.* 25:4891–4898.
41. Hildebrandt, S., Blaser, A., Parel, S.P., and Leumann, C.J. 1997. 5-substituted 2-aminopyridine C-nucleosides as protonated cytidine equivalents: increasing efficiency and selectivity in DNA triple helix formation. *J. Am. Chem. Soc.* 119:5499–5511.
42. Roberts, R.W., and Crothers, D.M. 1992. Stability and properties of double and triple helices: dramatic effects of RNA or DNA backbone composition. *Science.* 258:1463–1466.
43. Escude, C., Sun, J.S., Rougee, M., Garestier, T., and Helene, C. 1992. Stable triple helices are formed upon binding of RNA oligonucleotides and their 2'-O-methyl derivatives to double-helical DNA. *C. R. Acad. Sci. III.* 315:521–525.
44. Torigoe, H., Hari, Y., Sekiguchi, M., Obika, S., and Imanishi, T. 2001. 2'-O,4'-C-methylene bridged nucleic acid modification promotes pyrimidine motif triplex DNA formation at physiological pH: thermodynamic and kinetic studies. *J. Biol. Chem.* 276:2354–2360.
45. Kukreti, S., Sun, J.S., Garestier, T., and Helene, C. 1997. Extension of the range of DNA sequences available for triple helix formation: stabilization of mismatched triplexes by acridine-containing oligonucleotides. *Nucleic Acids Res.* 25:4264–4270.
46. Lacroix, L., et al. 1998. Triplex formation by oligonucleotides containing 5-(1-propynyl)-2'- deoxyuridine: decreased magnesium dependence and improved intracellular gene targeting. *Biochemistry.* 38:1893–1901.
47. Lacroix, L., Arimondo, P.B., Takasugi, M., Helene, C., and Mergny, J.L. 2000. Pyrimidine morpholino oligonucleotides form a stable triple helix in the absence of magnesium ions. *Biochem. Biophys. Res. Commun.* 270:363–369.
48. Arya, D.P., and Bruice, T.C. 1999. Triple-helix formation of DNA oligomers with methylthiourea-linked nucleosides (DNmt): a kinetic and thermodynamic analysis. *Proc. Natl. Acad. Sci. U. S. A.* 96:4384–4389.
49. Escude, C., et al. 1996. Stable triple helices formed by oligonucleoside N3' → P5' phosphoramidates inhibit transcription elongation. *Proc. Natl. Acad. Sci. U. S. A.* 93:4365–4369.
50. Dagle, J.M., and Weeks, D.L. 1996. Positively charged oligonucleotides overcome potassium-mediated inhibition of triplex DNA formation. *Nucleic Acids Res.* 24:2143–2149.
51. Ehrenmann, F., Vasseur, J.J., and Debart, F. 2001. Alpha-oligonucleotides with anionic phosphodiester and cationic phosphoramidate linkages enhanced stability of DNA triple helix. *Nucleosides Nucleotides Nucleic Acids.* 20:797–799.
52. Bijapur, J., Keppler, M.D., Bergqvist, S., Brown, T., and Fox, K.R. 1999. 5-(1-propargylamino)-2'-deoxyuridine (UP): a novel thymidine analogue for generating DNA triplexes with increased stability. *Nucleic Acids Res.* 27:1802–1809.
53. Rajeev, K.G., Jadhav, V.R., and Ganesh, K.N. 1997. Triplex formation at physiological pH: comparative studies on DNA triplexes containing 5-Me-dC tethered at N4 with spermine and tetraethyleneoxyamine. *Nucleic Acids Res.* 25:4187–4193.
54. Sund, C., Puri, N., and Chattopadhyaya, J. 1996. Synthesis of C-branched spermine tethered oligo-DNA and the thermal stability of the duplexes and triplexes. *Tetrahedron.* 52:12275–12290.
55. Cuenoud, B., et al. 1998. Dual recognition of double stranded DNA by 2'-aminoethoxy-modified oligonucleotides. *Angew. Chem. Int. Ed. Engl.* 37:1288–1291.
56. Blommers, M.J., Natt, F., Jahnke, W., and Cuenoud, B. 1998. Dual recognition of double-stranded DNA by 2'-aminoethoxy-modified oligonucleotides: the solution structure of an intramolecular triplex obtained by NMR spectroscopy. *Biochemistry.* 37:17714–17725.
57. Stutz, A.M., Hoeck, J., Natt, F., Cuenoud, B., and Woisetschlager, M. 2001. Inhibition of interleukin-4- and CD40-induced IgE germline gene promoter activity by 2'-aminoethoxy-modified triplex-forming oligonucleotides. *J. Biol. Chem.* 276:11759–11765.
58. Carlomagno, T., Blommers, M.J., Meiler, J., Cuenoud, B., and Griesinger, C. 2001. Determination of aliphatic side-chain conformation using cross- correlated relaxation: application to an extraordinarily stable 2'-aminoethoxy-modified oligonucleotide triplex. *J. Am. Chem. Soc.* 123:7364–7370.
59. Sollogoub, M., Dominguez, B., Fox, K.R., and Brown, T. 2000. Synthesis of a novel bis-amino-modified thymidine monomer for use in DNA triplex stabilization. *Chemical Communications.* 23:2315–2316.
60. Helene, C. 1991. The anti-gene strategy: control of gene expression by triplex-forming- oligonucleotides. *Anticancer Drug Des.* 6:569–584.
61. Rapozzi, V., et al. 2002. Antigene effect in K562 cells of a PEG-conjugated triplex-forming oligonucleotide targeted to the bcr/abl oncogene. *Biochemistry.* 41:502–510.
62. Puri, N., et al. 2001. Targeted gene knockout by 2'-O-aminoethyl modified triplex forming oligonucleotides. *J. Biol. Chem.* 276:28991–28998.
63. Faruqi, A.F., Datta, H.J., Carroll, D., Seidman, M.M., and Glazer, P.M. 2000. Triple-helix formation induces recombination in mammalian cells via a nucleotide excision repair-dependent pathway. *Mol. Cell Biol.* 20:990–1000.
64. Faruqi, A.F., Seidman, M.M., Segal, D.J., Carroll, D., and Glazer, P.M. 1996. Recombination induced by triple-helix-targeted DNA damage in mammalian cells. *Mol. Cell Biol.* 16:6820–6828.
65. Sandor, Z., and Bredberg, A. 1994. Repair of triple helix directed psoralen adducts in human cells. *Nucleic Acids Res.* 22:2051–2056.
66. Breitman, S., Amosova, O., Dolinnaya, N.G., and Fresco, J.R. 1999. Repairing the sickle cell mutation. I. Specific covalent binding of a photoreactive third strand to the mutated base pair. *J. Biol. Chem.* 274:21763–21768.
67. Vasquez, K.M., Dagle, J.M., Weeks, D.L., and Glazer, P.M. 2001. Chromosome targeting at short polypurine sites by cationic triplex-forming oligonucleotides. *J. Biol. Chem.* 276:38536–38541.

68. Vasquez, K.M., Narayanan, L., and Glazer, P.M. 2000. Specific mutations induced by triplex-forming oligonucleotides in mice. *Science.* 290:530–533.
69. Luo, Z., Macris, M.A., Faruqi, A.F., and Glazer, P.M. 2000. High-frequency intrachromosomal gene conversion induced by triplex-forming oligonucleotides microinjected into mouse cells. *Proc. Natl. Acad. Sci. U. S. A.* 97:9003–9008.
70. Chan, P.P., et al. 1999. Targeted correction of an episomal gene in mammalian cells by a short DNA fragment tethered to a triplex-forming oligonucleotide. *J. Biol. Chem.* 274:11541–11548.
71. Havre, P.A., and Glazer, P.M. 1993. Targeted mutagenesis of simian virus 40 DNA mediated by a triple helix- forming oligonucleotide. *J. Virol.* 67:7324–7331.
72. Havre, P.A., Gunther, E.J., Gasparro, F.P., and Glazer, P.M. 1993. Targeted mutagenesis of DNA using triple helix-forming oligonucleotides linked to psoralen. *Proc. Natl. Acad. Sci. U. S. A.* 90:7879–7883.
73. Vasquez, K.M., Wang, G., Havre, P.A., and Glazer, P.M. 1999. Chromosomal mutations induced by triplex-forming oligonucleotides in mammalian cells. *Nucleic Acids Res.* 27:1176–1181.
74. Lin, F.L., et al. 2000. Stability of DNA triplexes on shuttle vector plasmids in the replication pool in mammalian cells. *J. Biol. Chem.* 275:39117–39124.
75. Barre, F.X., et al. 2000. Unambiguous demonstration of triple-helix-directed gene modification. *Proc. Natl. Acad. Sci. U. S. A.* 97:3084–3088.
76. Giovannangeli, C., et al. 1997. Accessibility of nuclear DNA to triplex-forming oligonucleotides: the integrated HIV-1 provirus as a target. *Proc. Natl. Acad. Sci. U. S. A.* 94:79–84.
77. Belousov, E.S., et al. 1998. Triplex targeting of a native gene in permeabilized intact cells: covalent modification of the gene for the chemokine receptor CCR5. *Nucleic Acids Res.* 26:1324–1328.
78. Oh, D.H., and Hanawalt, P.C. 1999. Triple helix-forming oligonucleotides target psoralen adducts to specific chromosomal sequences in human cells. *Nucleic Acids Res.* 27:4734–4742.
79. Wang, G., Seidman, M.M., and Glazer, P.M. 1996. Mutagenesis in mammalian cells induced by triple helix formation and transcription-coupled repair. *Science.* 271:802–805.
80. Vasquez, K.M., Marburger, K., Intody, Z., and Wilson, J.H. 2001. Manipulating the mammalian genome by homologous recombination. *Proc. Natl. Acad. Sci. U. S. A.* 98:8403–8410.
81. Lin, F.L., Sperle, K., and Sternberg, N. 1990. Repair of double-stranded DNA breaks by homologous DNA fragments during transfer of DNA into mouse L cells. *Mol. Cell Biol.* 10:113–119.
82. Campbell, C.R., Keown, W., Lowe, L., Kirschling, D., and Kucherlapati, R. 1989. Homologous recombination involving small single-stranded oligonucleotides in human cells. *New Biol.* 1:223–227.
83. Gonez, K.K., Kunzelmann, K., Xu, Z., and Gruenert, D.C. 1998. Targeted replacement of normal and mutant CFTR sequences in human airway epithelial cells using DNA fragments. *Hum. Mol. Genet.* 7:1913–1919.
84. Datta, H.J., Chan, P.P., Vasquez, K.M., Gupta, R.C., and Glazer, P.M. 2001. Triplex-induced recombination in human cell-free extracts: Dependence on XPA and HsRad51. *J. Biol. Chem.* 276:18018–18023.
85. Gamper, H., Hou, Y.-M., Stamm, M.R., Podyminogin, M., and Meyer, R.B. 1998. Strand invasion of supercoiled DNA by oligonucleotide with a triplex guide sequence. *J. Am. Chem. Soc.* 120:2182–2183.
86. Biet, E., Maurisse, R., Dutreix, M., and Sun, J. 2001. Stimulation of RecA-mediated D-loop formation by oligonucleotide directed triple-helix formation: guided homologous recombination (GOREC). *Biochemistry.* 40:1779–1786.
87. Griffin, L.C., and Dervan, P.B. 1989. Recognition of thymine adenine base pairs by guanine in a pyrimidine triple helix motif. *Science.* 245:967–971.
88. Greenberg, W., and Dervan, P.B. 1995. Energetics of formation of sixteen triple helical complexes which vary at a single position within a purine motif. *J. Am. Chem. Soc.* 117:5016–5022.
89. Wang, E., Malek, S., and Feigon, J. 1992. Structure of a G.T.A triplex in an intramolecular DNA triplex. *Biochemistry.* 31:4838–4846.
90. Jiang, L., and Russu, I.M. 2001. Proton exchange and local stability in a DNA triple helix containing a G.TA triad. *Nucleic Acids Res.* 29:4231–4237.
91. Gianolio, D.A., and McLaughlin, L.W. 1999. Selective recognition of a dC-dG base pair by oligonucleotide directed triplex formation using a dC residue tethering an intercalator. *J. Am. Chem. Soc.* 121:6334–6335.
92. Griffin, L.C., Kiessling, L.L., Beal, P.A., Gillespie, P., and Dervan, P.B. 1992. Recognition of all four base pairs of double helical DNA by triple helix formation: design of nonnatural deoxyribonucleosides for pyrimidine-purine base pair binding. *J. Am. Chem. Soc.* 114:7976–7982.
93. Koshlap, K.M., Gillespie, P., Dervan, P.B., and Feigon, J. 1993. Nonnatural deoxyribonucleoside D3 incorporated in an intramolecular DNA triplex binds sequence-specifically by intercalation. *J. Am. Chem. Soc.* 115:7908–7909.
94. Prevot-Halter, I., and Leumann, C.J. 1999. Selective recognition of a C-G base-pair in the parallel DNA triple- helical binding motif. *Bioorg. Med. Chem. Lett.* 9:2657–2660.
95. Okiba, S., Hari, Y., Seltiguchi, M., and Imanishi, T. 2002. A 2′,4′-bridged nucleic acid containing 2-pyridone as a nucleobase: efficient recognition of a C-C interruption by triplex formation with a pyrimidine motif. *Angew. Chem. Int. Ed. Engl.* 40:2079–2081.
96. Huang, C.Y., Bi, G., and Miller, P.S. 1996. Triplex formation by oligonucleotides containing novel deoxycytidine derivatives. *Nucleic Acids Res.* 24:2606–2613.
97. Huang, C.Y., Cushman, C.D., and Miller, P.S. 1993. Triplex formation by an oligonucleotide containing N4-(3-acetamidopropyl)cytosine. *J. Org. Chem.* 58:5048–5049.
98. Rao, T.S., Hogan, M.E., and Revankar, G.R. 1994. Synthesis of triple helix forming oligonucleotides containing 2′-deoxyformycin. *Nucleosides Nucleotides.* 13:95–107.
99. Durland, R.H., et al. 1995. Azole substituted oligonucleotides promote antiparallel triplex formation at non-homopurine duplex targets. *Nucleic Acids Res.* 23:647–653.
100. Mokhir, A.A., Connors, W.H., and Richert, C. 2001. Synthesis and monitored selection of nucleotide surrogates for binding T·A base pairs in homopurine-homopyrimidine DNA triple helices. *Nucleic Acids Res.* 29:3674–3684.
101. Eldrup, A.B., Dahl, O., and Nielsen, P.B. 2002. A novel peptide nucleic acid monomer for recognition of thymine in triple helix structures. *J. Am. Chem. Soc.* 119:11116–11117.
102. Li, J.-S., Pan, Y.-H., Zhang, Y., Marky, L.A., and Gold, B. 2003. *J. Am. Chem. Soc.* 125:2084–2093.

MOLECULAR AND CELLULAR BIOLOGY, Feb. 2000, p. 990–1000 Vol. 20, No. 3
0270-7306/00/$04.00+0


# Triple-Helix Formation Induces Recombination in Mammalian Cells via a Nucleotide Excision Repair-Dependent Pathway

A. FAWAD FARUQI,[1] HIROCK J. DATTA,[1] DANA CARROLL,[2] MICHAEL M. SEIDMAN,[3] AND PETER M. GLAZER[1]*

*Departments of Therapeutic Radiology and Genetics, Yale University School of Medicine, New Haven, Connecticut 06520-8040[1]; Department of Biochemistry, University of Utah School of Medicine, Salt Lake City, Utah 84132[2]; and National Institute on Aging, National Institutes of Health, Baltimore, Maryland 21224[3]*

Received 31 August 1999/Returned for modification 13 October 1999/Accepted 11 November 1999

**The ability to stimulate recombination in a site-specific manner in mammalian cells may provide a useful tool for gene knockout and a valuable strategy for gene therapy. We previously demonstrated that psoralen adducts targeted by triple-helix-forming oligonucleotides (TFOs) could induce recombination between tandem repeats of a *supF* reporter gene in a simian virus 40 vector in monkey COS cells. Based on work showing that triple helices, even in the absence of associated psoralen adducts, are able to provoke DNA repair and cause mutations, we asked whether intermolecular triplexes could stimulate recombination. Here, we report that triple-helix formation itself is capable of promoting recombination and that this effect is dependent on a functional nucleotide excision repair (NER) pathway. Transfection of COS cells carrying the dual *supF* vector with a purine-rich TFO, AG30, designed to bind as a third strand to a region between the two mutant *supF* genes yielded recombinants at a frequency of 0.37%, fivefold above background, whereas a scrambled sequence control oligomer was ineffective. In human cells deficient in the NER factor XPA, the ability of AG30 to induce recombination was eliminated, but it was restored in a corrected subline expressing the XPA cDNA. In comparison, the ability of triplex-directed psoralen cross-links to induce recombination was only partially reduced in XPA-deficient cells, suggesting that NER is not the only pathway that can metabolize targeted psoralen photoadducts into recombinagenic intermediates. Interestingly, the triplex-induced recombination was unaffected in cells deficient in DNA mismatch repair, challenging our previous model of a heteroduplex intermediate and supporting a model based on end joining. This work demonstrates that oligonucleotide-mediated triplex formation can be recombinagenic, providing the basis for a potential strategy to direct genome modification by using high-affinity DNA binding ligands.**

One of the major goals of gene therapy for human disease is the targeted modification of the genome. Although methods for delivery of genes into mammalian cells are well developed, the frequency of homologous recombination is limited (22), in contrast to the more favorable situation in yeast. Most of the time, transfected DNA integrates in a nonhomologous manner, and, as a result, the simple introduction of new genes is of only modest value for gene therapy because it is difficult to achieve appropriate regulation of the gene when it is not in its cognate site. Techniques to select for homologous recombination of a sequence into the matching chromosomal locus have been developed (39), and these techniques have been invaluable in enabling the generation of mammalian cells and mice in which specific genes have been knocked out and replaced with desired sequences. However, such selection techniques require growth of cells in culture and are, in general, not applicable to gene therapy approaches. Because of this, investigators have recognized that methods to enhance the frequency of homologous recombination in mammalian cells will be critical in developing gene replacement strategies for research and gene therapy applications.

Recently, efforts have been directed toward modification of the recipient site to create a substrate prone to homologous recombination. It has been shown that a site-specific endonuclease, I-*Sce*I, can induce double-strand breaks within extrachromosomal and genomic DNA designed to carry the rare 18-bp recognition site. This strategy has been used to induce intermolecular and intramolecular recombination in both *Xenopus* oocytes (51) and mammalian cells (3, 10, 14, 27, 44, 47). However, this has limited general application, as it involves the prior introduction of the recognition site within the genome.

Besides double-strand breaks, other types of DNA damage have been shown to be recombinagenic, but in a non-site-specific way. These include DNA damage from UV radiation (54), chemical carcinogens (64), and photoreactive molecules such as psoralen (48). Psoralens are DNA-damaging agents that intercalate into DNA and form covalent monoadducts and interstrand cross-links upon exposure to near-UV light (UVA). Photo-induced psoralen interstrand cross-links, and to a lesser extent monoadducts, can induce recombination in bacteria (11, 37), yeast (48), and mammalian cells and viruses (21, 60).

In previous work, members of our group demonstrated that psoralen damage can be introduced into DNA within mammalian cells in a site-specific manner by linking psoralen to an oligonucleotide that is designed to form a triple helix (62). Triplex DNA can be formed when oligonucleotides bind in the major groove of the double helix in a sequence-dependent manner at polypurine-polypyrimidine stretches in duplex DNA (1, 12, 33, 41, 43). The specificity of oligonucleotide-mediated triplex formation has also been used to inhibit transcription and to cleave DNA at unique sites (24). Using this strategy, we found that triple-helix-targeted psoralen damage can generate site-specific mutations and mediate gene knockout within viral genomes replicating in monkey or human cells (17, 31, 62, 63)

* Corresponding author. Mailing address: Department of Therapeutic Radiology, Yale University School of Medicine, P.O. Box 208040, New Haven, CT 06520-8040. Phone: (203) 737-2788. Fax: (203) 737-2630. E-mail: peter.glazer@yale.edu.

and within chromosomal genes in mouse and hamster cells (38, 58).

Prompted by the ability of triple-helix-forming oligonucleotides (TFOs) to mediate genome modification in the form of mutation, we went on to test the ability of third-strand-targeted psoralen adducts to provoke homologous recombination at selected sites within cells. Using a simian virus 40 (SV40)-based shuttle vector carrying two mutant copies of the *supF* reporter gene, we demonstrated the ability of psoralen-conjugated TFOs to enter cells, to find and bind to their target site, and to stimulate intramolecular recombination within an episomal substrate (18). Other groups have also investigated recombination stimulated by triplex-directed psoralen adducts (50, 59). These studies have established directed psoralen adducts as potential tools to sensitize selected genomic sites to modification (7, 59), although the mechanism of this sensitization has not been worked out.

Based on these results, we sought in the present work to test whether triple-helix formation itself could stimulate homologous recombination. Previous work examining targeted mutagenesis had revealed that high-affinity third-strand binding, alone, could induce mutations in a target gene via a repair-dependent pathway (63). In that work, a substantial portion of the targeted mutations were deletions. An analysis of the deletion end points, presented here, reveals a pattern consistent with a pathway of strand break induction and end joining at regions of microhomology, suggesting that triplex formation might be able to generate intermediate structures containing strand breaks prone to homologous recombination. Using an SV40-vector-based assay, we report here the induction of homologous recombination in mammalian cells mediated by triple-helix formation even in the absence of an associated psoralen adduct or any other covalent damage. We find that the third-strand-stimulated recombination is dependent on nucleotide excision repair (NER), as triplex-induced recombination was not detected in cells deficient in the NER damage recognition factor, XPA, but was observed in a derivative line expressing the XPA cDNA. In comparison, we found that the recombination induced by the psoralen-triplex lesions was reduced only about 50% in the absence of NER, suggesting a partial but not exclusive role for NER in processing the targeted psoralen adducts into recombinagenic intermediates. The induced recombination, however, was unaffected in cells deficient in mismatch repair, prompting reevaluation of our previous model of an extensive heteroduplex intermediate and leading to a new model based on end joining.

## MATERIALS AND METHODS

**Vectors.** Plasmid pSupFAR was constructed to contain two mutant *supF* tRNA genes in tandem (Fig. 1). The two genes differ only at positions 115 and 163. This vector is similar to pSupF2 used in previous work of members of our group (18), except that the polymorphisms at positions 101 and 177 have been eliminated to simplify product analysis. The *supF* genes were derived from the genes in the vectors pSupFG1 (62) and pSP189 (42). pSupFG1 carries a novel *supF* gene modified to create a polypurine binding site for high-affinity triple-helix formation. The pSupFG1 derivative, pGW47, containing a C-to-G mutation at bp 163 within the *supFG1* gene (yielding the gene designated here as *supFT*), was digested with *Rsa*I. A mutant variant of the *supF* gene, carrying a G-to-A mutation at nucleotide position 115 (designated *supF2*), was generated as a synthetic 104-bp oligonucleotide fragment and inserted into the *Eco*RI-digested pGW47 DNA to yield the pSupFAR shuttle vector containing the two mutant *supF* genes as direct repeats separated by 9 bp. The orientation of the cloned fragment was confirmed by DNA sequencing. The vector also contains the SV40 origin of replication and large T-antigen coding sequence along with the β-lactamase gene and replication origin from pBR327, as described previously (18).

**Oligonucleotides.** Unconjugated and psoralen-linked oligonucleotides were synthesized by the Keck Facility at Yale University, by standard phosphoramidite chemistry with materials from Glen Research (Sterling, Va.). All oligomers contained phosphodiester backbones and were synthesized to contain a 3′ propylamine group to minimize susceptibility to degradation by 3′ exonucleases (26). The oligomers were purified by either gel electrophoresis or high-pressure liquid chromatography, followed by Centricon-3 filtration in distilled water (Amicon, Beverly, Mass.). The psoralen was incorporated into the oligonucleotide synthesis as a psoralen phosphoramidite, resulting in an oligonucleotide linked at its 5′ end via a two-carbon linker arm to 4′-hydroxymethyl-4,5′,8-trimethylpsoralen. The oligonucleotides used in this study were pso-AG30 (5′ psoralen-AGGAAG GGGGGGGTGGTGGGGGAGGGGGAG-3′), AG30 (same as pso-AG30 but without 5′ psoralen), pso-SCR30 (5′ psoralen-GGAGGAGTGGAGGGGAGT GAGGGGGGGGGG-3′), and SCR30 (same as pso-SCR30 but without 5′ psoralen).

**Cells.** Monkey COS-7 cells were obtained from the American Type Culture Collection (1651-CRL). Transformed XPA fibroblasts from patient XP2OS and the XP2OS cells transfected with a vector expressing XPA cDNA [XP2OS(pCAH19WS)] were obtained from K. Kraemer (34). XPF and XPG fibroblasts (from patients with xeroderma pigmentosum, groups F and G) were obtained from the National Institute of General Medical Science Human Genetic Mutant Cell Repository (Camden, N.J.), repository no. GM08437 and GM03021B, respectively. The XPF cells were obtained as an SV40-transformed fibroblast line; the XPG cells are a primary fibroblast culture obtained at passage 10. The human colon cancer cell line, HCT116, deficient in the DNA mismatch repair (MMR) factor MLH1, along with a subline corrected by whole-chromosome transfer to restore MMR function (HCT116.3-6, corrected with chromosome 3) and a control subline (HCT116.2-3, with chromosome 2), were obtained from T. Kunkel (28). A similar set of MSH2-deficient and corrected cell lines, HC (MSH2 deficient), HC.2-4 (corrected with chromosome 2), and HC.7-2 (control with chromosome 7), were also obtained from T. Kunkel (55). The cells were grown in growth media (Dulbecco's modified Eagle's medium [Life Technologies, Gaithersburg, Md.], 10% fetal calf serum [Life Technologies], and 1% penicillin–1% streptomycin (Sigma, St. Louis, Mo.)] at 37°C in a humidified incubator in the presence of 5% $CO_2$.

**Intracellular targeting and recombination assay.** In the case of the COS cells, subconfluent cells were detached by trypsinization and washed three times in growth medium. The cells were resuspended at a density of $10^7$ cells/ml, and pSupFAR plasmid DNA was added at a concentration of 3 μg of DNA/$10^6$ cells. The cell-DNA mixture was then incubated on ice for 10 min. Transfection of the cells was carried out by electroporation with a gene pulser (Bio-Rad, Hercules, Calif.) at a setting of 25 μF/250 W/250 V in 0.4-cm gap transfection cuvettes (Bio-Rad). After 10 min of incubation at room temperature, cells were washed twice in growth medium to remove excess extracellular plasmid DNA and replated in culture in growth media into 100-mm-diameter dishes. After 24 h, cells were transfected with selected oligonucleotides by electroporation at a concentration of 2 μM in the cell suspension, as described above, and UVA irradiation, if indicated, was administered 2 h later. In the case of human cell lines (XPA, XPA corrected, XPF, and XPG), the shuttle vector DNA was transfected by using Lipofectamine (Life Technologies), with a ratio of 3 μg of plasmid DNA to 30 μl of Lipofectamine, premixed with 600 μl of Dulbecco's modified Eagle's medium, and was added to cells with 2.4 ml of medium in 60-mm-diameter dishes, as directed by the manufacturer. After 24 h, the oligonucleotides were transfected into the cells with Lipofectamine, with an oligonucleotide-to-lipid ratio of 6 μg to 60 μl in a 600-μl volume.

For all cell types, 48 h after transfection the cells were harvested for plasmid DNA isolation by a modified alkaline lysis procedure (62), and the resulting vector DNA was subjected to digestion with *Dpn*I (to eliminate any unreplicated molecules that had not acquired the mammalian methylation pattern) and used to transform bacteria for genetic analysis of *supF* gene function as previously described (62). Selected colonies were purified, and the plasmids were isolated for PCR or DNA sequence analysis.

**Product analysis.** Screening of the recombinants by PCR amplification of the sequences containing the *supF* gene(s) in the vectors was performed with the following primers: 5′ GGCGACACGGAAATGTTGAA 3′ (forward) and 5′ TTAGCTTTCGCTAAGGATCCGG 3′ (reverse). DNA sequencing was carried out as described previously (62). The sequencing primer was the same as the forward primer used for screening of the recombinants, listed above.

**In vitro triplex binding, photoadduct formation, and cotransfection shuttle vector assay.** In a reaction volume of 10 μl, 3 μg (50 nM) of the pSupFAR DNA was incubated with a 100:1 molar excess of oligonucleotide (5 μM) for 2 h at 37°C in 10% sucrose, 20 mM $MgCl_2$, 10 mM Tris (pH 8.0), and 1 mM spermidine (triplex binding buffer). If indicated, irradiation of samples with 1.8 J/$cm^2$ of UVA was performed using 365-nm lamps supplied by Southern New England Ultraviolet Co. (Branford, Conn.). A radiometer (International Light, Newburyport, Mass.) was used to measure the lamp output (typical UVA irradiance of 5 to 7 mW/$cm^2$ at 320 to 400 nm). A window glass filter was used to eliminate any UVB contamination during the UVA irradiation. Cotransfection of the preformed oligonucleotide-plasmid DNA complexes into the human repair-deficient and -proficient cell lines was carried out using Lipofectamine (Life Technologies), as described above. After 48 h, shuttle vector isolation and analysis was performed as described above.

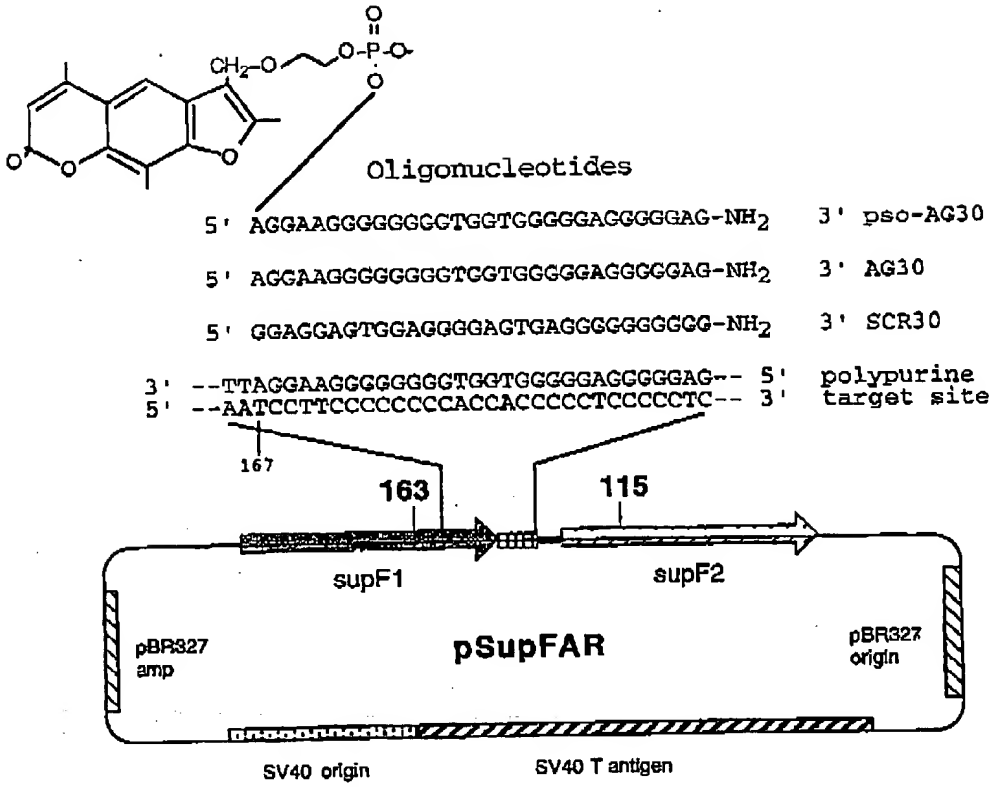



FIG. 1. Schematic representation of the pSupFAR vector. The SV40-based shuttle vector contains two mutant *supF* genes in the form of a tandem dimer. The upstream mutant *supF* gene, *supF1*, contains a C-to-G point mutation at nucleotide position 163; the downstream mutant *supF* gene, *supF2*, contains a G-to-A point mutation at nucleotide position 115. The two *supF* genes are separated by a 9-bp sequence that contains an *EagI* restriction site used for cloning. At the 3' end of *supF1* is an engineered polypurine sequence (bp 167 to 196), creating a high-affinity third-strand binding site. A purine-rich oligonucleotide with a length of 30 nucleotides (AG30) was designed to form a triple helix in the anti-parallel triplex motif at this site, as shown. As a control, SCR30, containing the same base composition but a scrambled sequence, was used. In some experiments, the AG30 and the SCR30 oligonucleotides were conjugated at their 5' end to 4'-hydroxymethyl-4,5',8-trimethylpsoralen via the 4' hydroxymethyl position, as shown in the case of pso-AG30. In this case, by formation of the triple helix, psoralen intercalation is targeted to the duplex-triplex junction at bp 166 to 167 of *supF1*. Upon photoactivation with UVA, both monoadducts and interstrand cross-links are generated at the thymidines in these base pairs.

## RESULTS

**Design of vector to detect induced recombination.** The vector pSupFAR was constructed to investigate the ability of triplex-directed DNA damage to trigger recombination between tandem *supF* genes flanking the third-strand binding site (Fig. 1). *supF* encodes an amber suppressor tyrosine tRNA whose function can be screened by a colorimetric assay in host bacteria carrying a *lacZ* nonsense mutation. To facilitate the detection of recombinants via a phenotypic screen, the upstream *supF1* gene in the vector carried a C-to-G mutation at bp 163, whereas the downstream *supF2* gene was engineered to contain a G-to-A mutation at position 115. Both of these changes disrupt *supF* activity. Hence, recombination between *supF1* and *supF2* has the potential to reconstruct a fully functional *supF* gene, which can be identified by the resulting blue colonies on indicator plates. (This vector differs slightly from the pSupF2 vector used in our previous study of psoralen-triplex-induced recombination (18) in that the sequence differences between the two *supF* genes at positions 101 and 177 have been eliminated, a situation which in the previous work reduced the yield of detectable wild-type recombinants).

The 30-bp polypurine-polypyrimidine site in the vector at the 3' end of *supF1* is a good target for triplex formation by the G-rich oligonucleotide, AG30 (Fig. 1). This oligomer was shown to bind to the target sequence with high affinity (equilibrium dissociation constant [$K_d$], $10^{-8}$ M) (63). In some of the experiments reported here, a derivative of this TFO was used; the derivative was synthesized to contain a psoralen conjugate at its 5' end, yielding pso-AG30, such that the third-strand binding positions the tethered psoralen for intercalation and adduct formation at bp 166 to 167 of the *supF1* gene (Fig. 1). The binding of the modified pso-AG30 to this site was determined to have a $K_d$ of $3 \times 10^{-9}$ M (62). The level of binding by AG30 and pso-AG30 was previously found to be sufficient for intracellular triplex formation and targeted mutagenesis in a shuttle vector carrying the *supFG1* gene (62, 63). As a control, a scrambled sequence oligonucleotide, SCR30, was used. It is a G-rich 30-mer and has the same base composition as AG30, but it has 14 mismatches in the third-strand binding code relative to the polypurine site in the pSupFAR vector, whereas AG30 has only two mismatches. No binding of SCR30 to the *supF* target site is detectable in gel mobility shift assays up to a concentration of $10^{-6}$ M.

**Triplex-induced recombination in COS cells.** To test the possibility that intermolecular triple-helix formation, by itself, might promote recombination, we examined the activity of

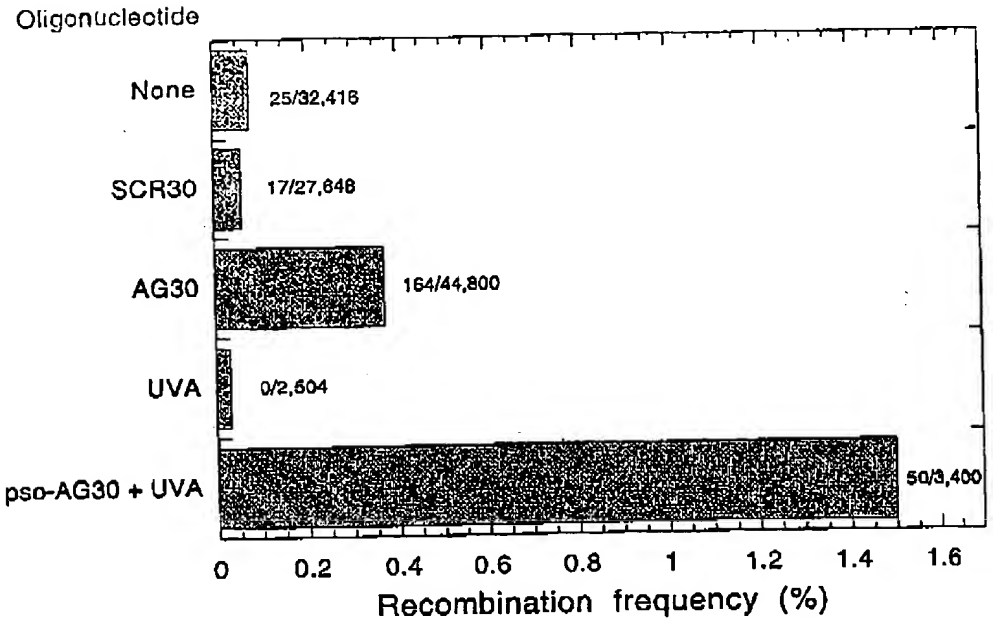



FIG. 2. Triplex-induced recombination in COS cells. Cells pretransfected with the pSupFAR vector were subsequently transfected with the indicated oligonucleotides. After 48 h, shuttle vector DNA was isolated from the cells for analysis of *supF* gene function and quantification of recombination events. The number of blue colonies (representing recombinants) out of the total number of colonies is presented to the right of each bar, with the bars indicating recombination frequency. One sample received only irradiation with UVA light (1.8 J/cm$^2$ of broad band long wavelength UV light centered at 365 nm) and no oligonucleotide, as indicated. In the case of pso-AG30 plus UVA, the irradiation was administered 2 h after TFO transfection.

AG30 in the pSupFAR recombination assay. COS cells were transfected with pSupFAR and subsequently transfected with either AG30, pso-AG30, or, as a sequence control, SCR30. In the case of pso-AG30, 2 h after oligonucleotide transfection, the cells were irradiated with long wavelength UVA light (365 nm) to activate the tethered psoralen for photoadduct formation. The treated cells were maintained in culture for 48 h before rescue of the vector DNA for analysis.

Recombinant colonies were identified by their nonparental blue phenotype among the parental white ones, the colonies were counted, and the recombination frequency was determined (Fig. 2). Cells not exposed to any oligonucleotide yielded a background frequency of 0.08% blues. When cells were treated with pso-AG30 followed by UVA, blue colonies were generated at a frequency of 1.5%, consistent with the previous work of members of our group (18). When AG30, lacking any psoralen conjugate, was used, recombinants were generated at a frequency of 0.37%, fivefold above background. SCR30 yielded only background levels of recombinants (0.06%). Hence, triplex formation, itself, is able to provoke recombination, at a level in this assay about 25% of that induced by targeted psoralen-triplex lesions.

**Analysis of nonparental products.** To ascertain the nature of the oligomer-induced events, we used PCR analysis to determine the *supF* gene copy number in the nonparental plasmids. Plasmid DNA from selected blue colonies, along with DNA from parental white ones for comparison, was amplified with primers flanking the tandem *supF* genes in pSupFAR. Analysis of the blue colonies produced by both AG30 and pso-AG30 revealed that all ($n$ = 22 and 12, respectively) were recombinants in which only a single, wild-type *supF* gene was present (data not shown; this interpretation was confirmed by DNA sequencing of 10 randomly selected recombinant vectors), whereas the white colonies contained the parental plasmid with two *supF* genes. Hence, all the blue recombinants tested appear to have arisen from nonconservative events involving the loss of sequences in which a functional *supF* gene was generated.

**Repair dependence of triplex-induced recombination.** To address the mechanism by which an intermolecular triple helix could promote recombination, we carried out the shuttle vector recombination assay in a repair-deficient human cell line (XP2OS) derived from a patient with xeroderma pigmentosum, group A (34). The XPA protein is a critical damage recognition factor in the NER pathway (49), and in previous work of members of our group, cells without XPA function were found to lack TFO-induced mutagenesis (63). In comparison, an otherwise isogenic subclone corrected to normal NER function by transfection with a vector expressing the wild-type XPA cDNA was used (34). In these experiments, the cells were transfected with pSupFAR on day 1 and with the oligomers on day 2, with cationic lipids being used both days. (The transfection protocol differed from that used for COS cells because of the poor survival of the XPA cells after electroporation). The oligonucleotides tested were AG30, pso-AG30, and SCR30. However, UVA irradiation was not performed, so in this experiment the comparison between AG30 and pso-AG30 reflects the possible effect of the psoralen conjugate only as an intercalator, not as a covalent adduct or interstrand cross-link.

The data (Fig. 3) reveal that AG30 and pso-AG30 (without UVA and hence without cross-linking) were effective in stimulating recombination in pSupFAR, both at frequencies of 0.14%, but only in the XPA cells that had been corrected to normal or near normal NER activity by expression of the XPA cDNA. In the mutant XPA cell line, no TFO-induced recom-

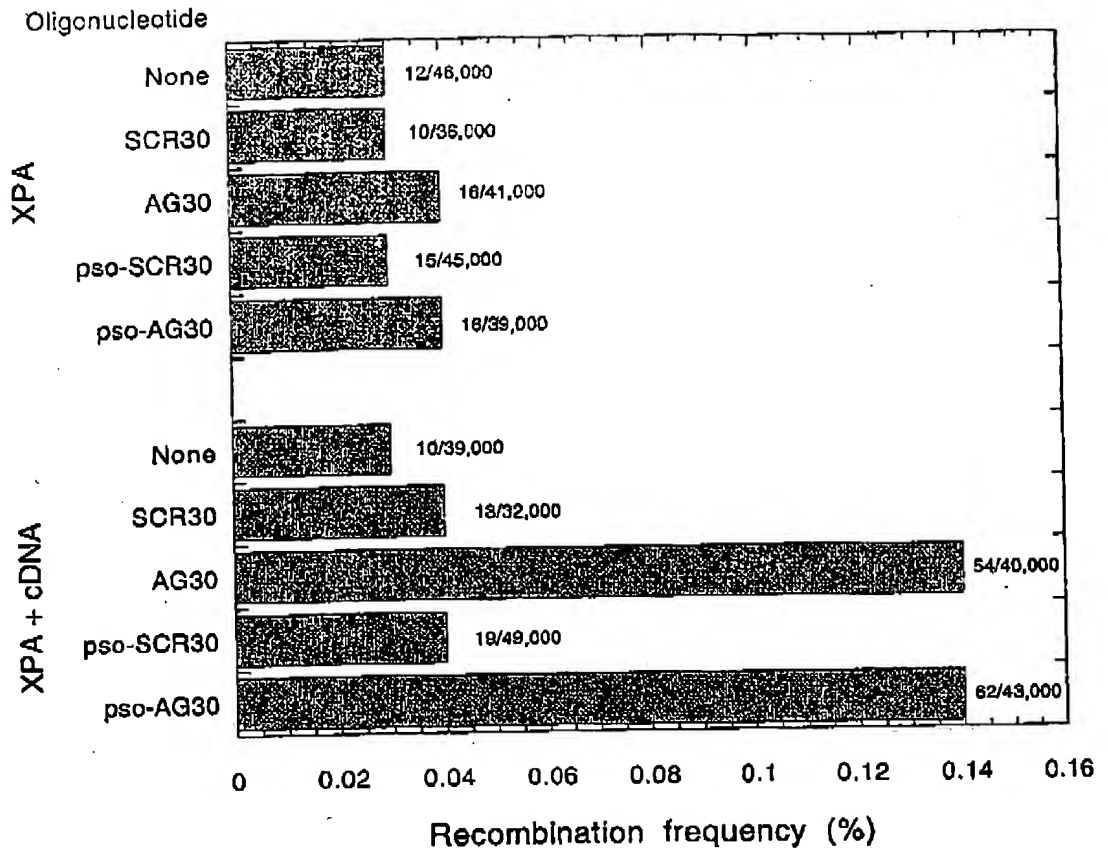



FIG. 3. Triplex-induced recombination in human repair-proficient and repair-deficient cells. The cells, either XPA (XP20S, deficient in the damage recognition factor XPA) or XPA corrected [XP20S(pCAH19WS) cells, a subline of XP2OS expressing wild-type XPA cDNA], were pretransfected with the pSupFAR vector and subsequently transfected with the indicated oligonucleotides. After 48 h, shuttle vector DNA was recovered and analyzed as described for Fig. 2.

bination was detected above the background (0.03%). Hence, the results show that the ability of an intermolecular triple helix to stimulate recombination requires an intact NER pathway, presumably to generate a recombinagenic intermediate. Interestingly, pso-AG30 without UVA (no covalent adducts) was not more effective than AG30, suggesting that the potential intercalation at the duplex-triplex junction by psoralen does not have a major influence on this process, and that the primary lesion that induces recombination is the triple helix itself. As in the COS cell experiments, all the analyzed blue colonies produced by AG30 were single gene recombinants ($n = 14$).

Note that the TFO-induced recombination frequencies in the XPA-corrected cells are slightly lower than that seen with AG30 in the COS cells (Fig. 2). This difference could have several explanations, as follows: (i) the NER correction may be incomplete; (ii) the COS cells have a more robust recombination activity; or (iii) the transfection of the TFOs into the COS cells was more effective. None of these would invalidate the basic observations and conclusions.

**Partial NER dependence of psoralen-triplex-induced recombination.** For comparison, we examined the role of the NER pathway in the recombination induced by psoralen-triplex lesions. In these experiments, the triple helices were formed in vitro on the vector DNA and the complexes were UVA irradiated to generate covalent psoralen adducts prior to transfection into the cells. This protocol was carried out to avoid any potential confounding variability in cellular oligonucleotide uptake and intracellular triplex formation that might arise because two unrelated, nonisogenic cell lines were included in the comparison. In this way, all the cell lines were challenged with a uniform DNA substrate. Also, this protocol avoids any possible toxicity to the repair-deficient cells from the UVA.

In prior studies (20, 61, 62), we demonstrated that under the conditions used here, the TFO-tethered psoralen covalently reacts with both strands of the duplex (via photoaddition at both the pyrone and furan rings to T's in opposite strands) to generate interstrand cross-links. In this structure, all three strands of the triplex are covalently linked via the psoralen. The TFO is connected through the linker arm attached to the 5′ end of the TFO and to the 4′-hydroxymethyl group of the psoralen. The duplex strands are linked via cyclobutane bonds between thymidines and the pyrone and furan rings of the psoralen.

The preformed pSupFAR-triplex-psoralen adduct complexes were transfected into a series of repair-deficient and -proficient cells, including not only the XPA cells and those of their corrected subclone, but also those of cell lines derived from xeroderma pigmentosum patients in complementation groups F and G. (The XPA and XPF cell lines were SV40

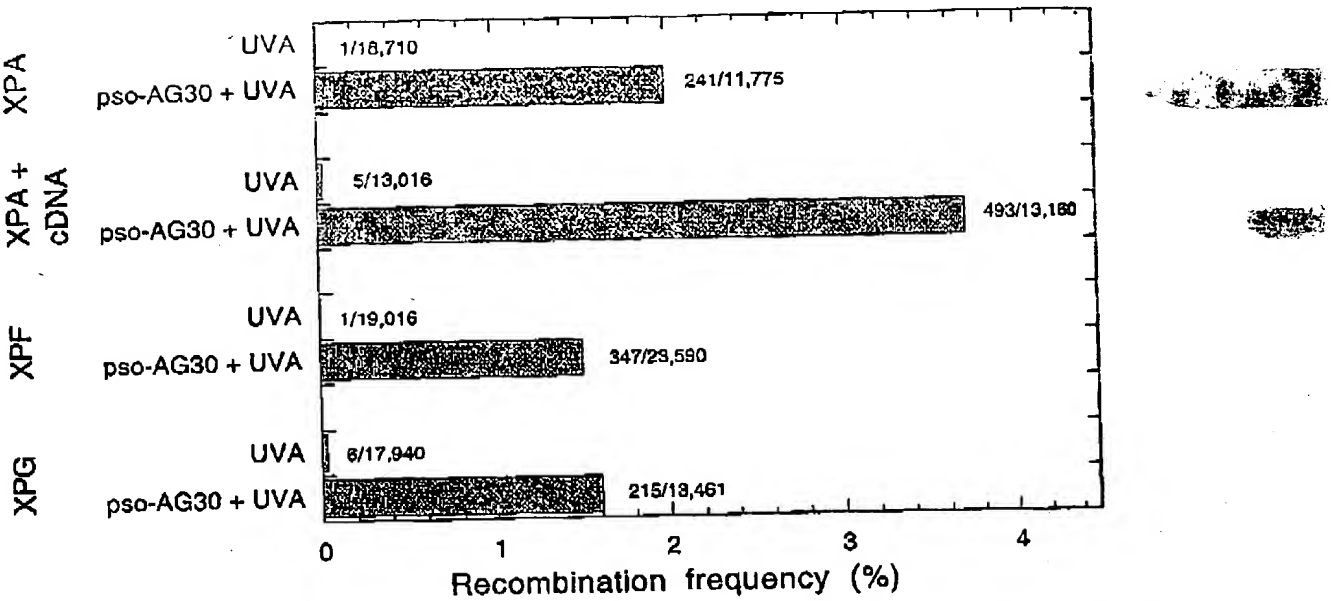



FIG. 4. Recombination induced by triplex-directed psoralen adducts in human repair-proficient and repair-deficient cells. Cells included either XPA (XP2OS, deficient in the NER damage recognition factor XPA) or XPA corrected [XP2OS(pCAH19WS) cells, a subline of XP2OS expressing wild-type XPA cDNA], along with cells from patients with xeroderma pigmentosum groups F and G, both of which are deficient in specific endonuclease activities required for NER. As indicated, the pSupFAR vector DNA was incubated in vitro with pso-AG30, followed by UVA irradiation to generate triplex-directed, psoralen photoadducts. The vector-triplex-psoralen adduct complexes were transfected into the indicated cells. Control samples included vector DNA irradiated with UVA in the absence of any oligonucleotide and then transfected directly into the various cell lines, as indicated. After 48 h, shuttle vector DNA was recovered and analyzed for recombination as described for Fig. 2.

transformed; the XPG cells were a primary fibroblast culture at passage 10.) XPF and XPG proteins play central roles in the endonuclease incision steps in NER, at positions 5' and 3' to the lesion, respectively (49). The data (Fig. 4) show that the covalent psoralen-triplex lesions stimulate recombination to a substantial degree even in the three NER-deficient cell types, XPA (2.0%), XPF (1.5%), and XPG (1.6%). However, a higher overall frequency of induced recombination was observed in the corrected XPA cells (3.7%). These results suggest that some but not all of the recombination induced by the psoralen-triplex compound lesion depends on NER. This conclusion is consistent with the emerging concept that non-NER pathways exist in cells that can metabolize psoralen cross-links. Furthermore, the observation that, as in the COS cells (Fig. 2), the covalent psoralen-triplex lesions (Fig. 4) are more effective than the triplex alone (Fig. 3) in stimulating recombination is also in keeping with the possibility that the psoralen cross-links are subject to metabolism by repair pathways in addition to NER.

**Triplex-induced recombination is mismatch repair independent.** To further extend our mechanistic understanding of the TFO-induced recombination, we tested the ability of pso-AG30 plus UVA to promote recombination in cells deficient in DNA MMR. Two human cancer-derived cell lines, HCT116 and HC, defective in MLH1 and MSH2, respectively were tested in comparison to subclones corrected to MMR proficiency by chromosome transfer, along with control sublines carrying noncorrecting chromosomes (28, 55). No differences in the yields of recombinants were detected whether or not the cells were MMR proficient or deficient (Fig. 5). (A difference was seen between the level of recombination in the HCT116-derived cells versus the HC cells. However, these differences do not correlate with MMR capacity. They may reflect differences in other, as yet unidentified repair or recombination factors, but this remains to be determined.) In these experiments, analysis of the induced blue colonies revealed single gene recombinants regardless of whether they were produced in MMR mutant or wild-type cells (data not shown).

These results call into question a previous model invoking a heteroduplex intermediate that is resolved by mismatch repair (18). The possibility remains that such an intermediate is, in fact, formed but is resolved by a mismatch repair pathway that is MSH2 and MLH1 independent. However, another possibility is that the triplexes promote recombination via a pathway in which strand breaks are produced (either by NER in the case of the triplexes alone or NER plus some other repair activity in the case of the psoralen-triplex lesion), and that these strand breaks lead to end-joining events at regions of microhomology.

**Triplex-induced deletion mutations.** If such a pathway of strand break production and end joining is provoked by triplex formation, we reasoned that the triplex-induced deletions found in our previous work (62, 63) might also provide evidence consistent with an end-joining mechanism. In our previous COS cell experiments, such deletions typically constituted about 15 to 25% of the observed mutations, yielding deletion frequencies in the range of 0.1 to 0.5% (for triplex alone and for covalent psoralen-triplexes, respectively), values which are comparable to the frequencies of induced recombinants seen in the work reported here (compare the COS cell data in Fig. 2). In Fig. 6, an analysis of randomly selected deletion mutations generated by psoralen-triplexes (Fig. 6A) and by intermolecular triple helices alone (Fig. 6B) is shown. In both data sets, most of the deletion end points appear to have been joined at sites of 3 to 4 bp of homology, in keeping with an end-joining model. However, unlike the *supF* recombinants in the experiments described above, the joints in the

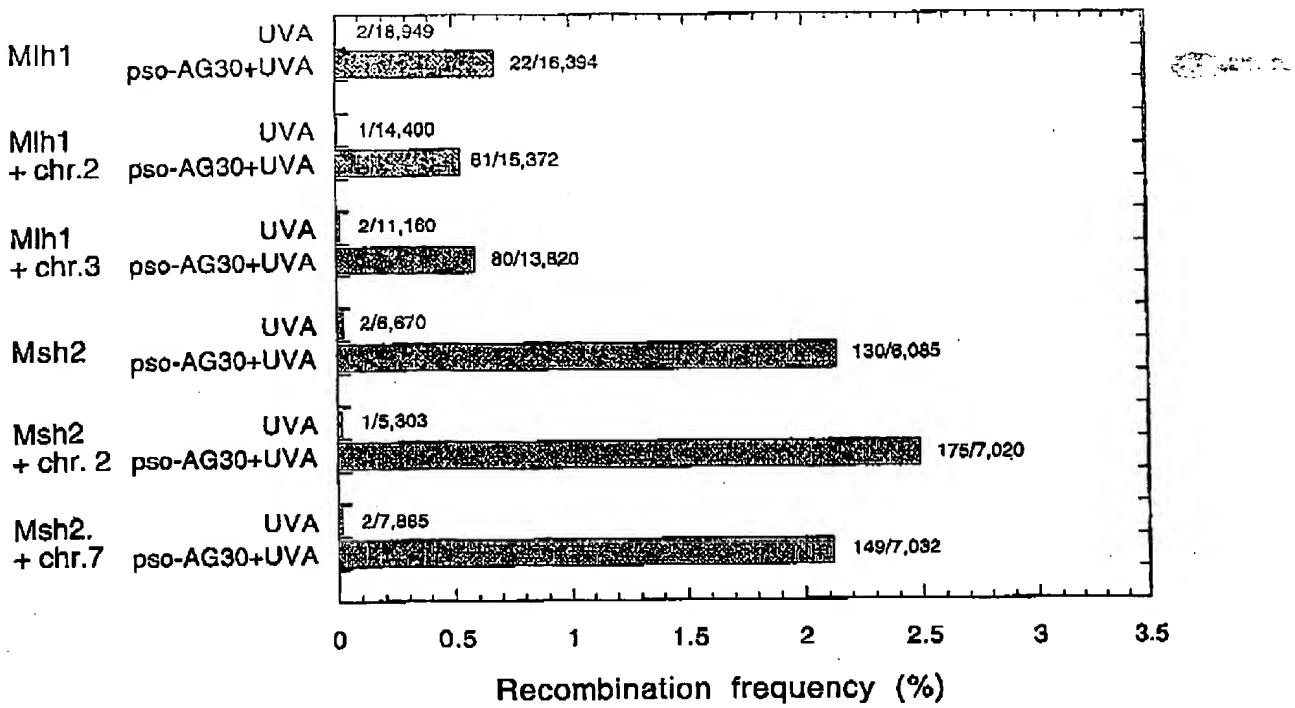



FIG. 5. Recombination induced by triplex-directed psoralen adducts in human DNA MMR-deficient and -proficient cells. Cells included the human colon cancer cell line, HCT116, deficient in the MMR factor MLH1, along with a subline corrected by whole-chromosome [chr.] transfer to restore MMR function (HCT116.3-6, corrected with chromosome 3) and a control sub-line (HCT116.2-3, with chromosome 2). A similar set of MSH2-deficient and corrected cell lines, HC (MSH2 deficient), HC.2-4 (corrected with chromosome 2), and HC.7-2 (control with chromosome 7) were also compared. The pSupFAR vector DNA was incubated in vitro with pso-AG30, followed by UVA irradiation to generate triplex-directed, psoralen photoadducts. The vector-triplex-psoralen adduct complexes were transfected into the indicated cells. Control samples included vector DNA irradiated with UVA in the absence of any oligonucleotide and then transfected directly into the various cell lines, as indicated. After 48 h, shuttle vector DNA was recovered and analyzed for recombination as described for Fig. 2.

deletions otherwise occur within surrounding regions of nonhomology. Also, there is one example in each set that has no homology around the deletion. These products are consistent with previous analyses of joints made in mammalian cells with ends produced intentionally by restriction enzymes (46).

## DISCUSSION

The work presented here demonstrates that intermolecular triple-helix formation can provoke a pathway of DNA metabolism that can lead to recombination. Cells pretransfected with an SV40-based shuttle vector carrying two mutant copies of the *supF* gene were subsequently transfected with specific TFOs, and it was found that such oligomers could provoke recombination within the vector in a sequence-dependent manner. The reaction was sequence specific, because only the AG30 or pso-AG30 oligonucleotides, which bind with high affinity to the target site, were found to generate recombinant products in the cells. The scrambled sequence oligomer, SCR30, was not active in the assay.

Experiments with NER-deficient cells lacking XPA function and with a corrected, otherwise isogenic subline reveal that NER is required for production of the recombinants by the triple helices. This finding is consistent with our previous observation that high-affinity third-strand binding can stimulate DNA repair activity in human cell extracts and can induce mutations in a target gene in human cells in an NER-dependent manner (63). The combined results provide strong support for the hypothesis that the formation of an intermolecular triple helix by an oligonucleotide is recognized by the NER pathway as a form of DNA damage, putting triplexes in a broad category of NER-processed lesions with pyrimidine dimers and bulky adducts such as those from acetyl-aminofluorene and benzo[*a*]pyrene. It is likely that further studies will reveal common structural features shared among triplexes and the other types of DNA damage that promote NER recognition. Furthermore, by extension from these results, it is expected that other classes of noncovalent DNA binding ligands, such as polyamides (65) and peptide nucleic acids (15, 16), may create alterations of DNA structure that will be recognized and metabolized by NER. As a result, TFOs, polyamides, peptide nucleic acids, and similar ligands may prove to be useful reagents in strategies aimed at harnessing the cell's repair mechanisms to promote genome modification. However, the particular properties of these various ligands in this regard remain to be determined.

The necessity for NER in the triplex-induced recombination also supports our previous study of gene correction with TFOs covalently linked to short "donor" fragments of DNA (8). In that work, the site-specific binding of the TFO domain of the hybrid molecule was designed to enhance the homology search and thereby accelerate recombination or gene conversion. The results reported here further support the notion that the TFO domain can also promote recombination of the donor fragment with the target site by recruiting the NER apparatus to

A

```
GTT GGA [ATC...CCC GGA] TCC TT
GGA ATT [CGG...GAA ATT] CGG TA
ATT CGG [AGA...ACC CGG] TAC CG
AAT TCG [AGA...AAT TCG] GTA CC
CTG CCG [TCA...TTA GCG] AAA GC
GTT GGA [ATC...CCC GGA] TCC TT
AAT TCG [AGA...TAC CCG] GTA CC
GAA AGC [ATT...CCA CCA] CCA CG
```

B

```
AGG GAA [TTC...CCC GAA] AGC TA
CCC TGC [TCC...GCC AAA] GGC AG
GCT CGA [GCT...CTT CGA] AGG TT
CAT CGA [CTT...GTT CGA] ATC CT
GAA TCC [TTC...CCC TCC] CCC TC
GAA TTC [GAG...TCC TTC] CCC CC
GAC TTC [GAA...TCC TTC] CCC CC
```

FIG. 6. Sequence analysis of deletion mutations generated in the *supF* gene in COS cells by triplex-associated psoralen adducts (A) and by triple helix formation alone (B). The deleted sequences are indicated within the brackets. Underlined nucleotides indicate stretches of microhomology at the deletion end points.

the target to create strand breaks and recombinagenic intermediates.

The role of the NER pathway in recombination stimulated by triplex-directed psoralen cross-links was also examined. In the case of the triplex-psoralen lesion, however, there was only a partial requirement for NER. About 40 to 50% of the induced recombination was found to be independent of NER function. This observation is in keeping with a study by Hall and Scherer (21) in which reactivation of psoralen and UVA-treated herpes simplex virus was seen in XPA-deficient cells when the cells were infected at high multiplicity, suggesting that a recombinational repair pathway was still active in these cells even in the absence of XPA function. This observation is also consistent with recent studies providing further evidence for non-NER repair of psoralen cross-links in mammalian cells. Li et al. found that psoralen cross-links but not monoadducts in one plasmid can stimulate repair synthesis in a second, undamaged plasmid in a human whole-cell extract (35). This activity was found to be separate from NER, as it was observed in extracts of several NER-deficient cell lines (XPA-, XPC-, and XPG-deficient cells).

Interestingly, the activity observed by Li et al. (35), while present in extracts from XPA cells, was absent from XPF cell extracts. This finding fits with earlier observations of an extreme hypersensitivity of XPF (ERCC4) mutant CHO cells to cross-linking agents (4, 25). Evidence suggests that, of the known NER factors, the XPF-ERCC1 heterodimer complex appears to have a special role in cross-link repair (4). Yet, our results suggest that even in XPF-deficient cells, pso-TFO-mediated cross-links can promote recombination, raising the additional possibility that either (i) another factor or set of factors may act on pso-TFO lesions in a recombinational repair pathway or (ii) the ability of pso-TFO-directed cross-links to block replication (rather than provoke repair) may play a role in stimulating recombination.

In other work looking more directly at cross-link repair in vitro, Reardon et al. showed that psoralen monoadducts and cross-links can be removed from a DNA substrate in human cell extracts (45). However, Bessho et al. (2) reported that purified NER factors reconstituted in vitro that can otherwise carry out excision repair of pyrimidine dimers cannot excise psoralen cross-links. In fact, they observed the production of dual incisions on one strand on the same side of the lesion rather than the expected flanking incisions (2), representing essentially a situation of incomplete repair. While such a strand break product may constitute an intermediate potentially prone to recombinational repair, this observation suggests that the known NER factors are insufficient for full psoralen cross-link repair in mammalian cells. Interestingly, the situation in *Escherichia coli* appears to be different, as NER factors do make dual incisions flanking the psoralen cross-link and are required for mediating cross-link repair, in a model elaborated by the work of Cole (11), Sladek et al. (53), and others (9, 56, 57).

Such subtle but important differences in the way psoralen lesions are repaired may help to explain why triplex-directed psoralen adducts were relatively ineffective at stimulating recombination in *Xenopus* oocytes compared with human or monkey cells (52). In the *Xenopus* system, the dual *supF*-carrying plasmid containing triplex-psoralen adducts showed little recombination (52), while similar substrates containing restriction enzyme-induced double-strand breaks recombined at high efficiency (51).

Experiments with MMR-deficient cells provided the unexpected result that MMR activity, at least the activity dependent on MSH2 and MLH1, is not required for the induced recombination in the pSupFAR shuttle vector. This result calls into question some aspects of our previous working model (18), in which we proposed that the psoralen-triplex-induced recombination takes place via a single-strand annealing pathway (18, 36). In such a pathway, damage-induced strand breaks in the substrate DNA would be followed by exonuclease activity to expose mostly homologous single-stranded regions that can anneal to form heteroduplexes. We had proposed the creation of an extensive heteroduplex intermediate, requiring resolution via MMR to generate the observed recombination products.

The lack of an effect of MSH2 and MLH1 in our assay is subject to several interpretations, as follows. (i) A heteroduplex intermediate is formed but is metabolized by an MSH2- and MLH1-independent pathway. The formation of such heteroduplexes in extrachromosomal recombination substrates carrying duplicated genes has been demonstrated (5, 13, 32, 40), so we cannot rule out this possible explanation. (ii) The triplex-targeted damage is metabolized through an end-joining pathway in which double-strand breaks are produced, but followed first by a more limited exonuclease activity and then by annealing of the ends at relatively short segments of homology. Such a pathway, diagrammed in Fig. 7, could eliminate the mutations at positions 163 and 115 and construct a functional *supF* gene without necessitating the production of a heteroduplex. A similar mechanism could account for the joints formed in the deletion mutations shown in Fig. 5, as these appear to have occurred by joining at short (3 to 4 bp) stretches of homology. In this model, however, a distinction should be made between the deletion and the recombination events. The deletions are proposed to arise from triplex-induced strand breaks that undergo illegitimate recombination and end joining at regions of microhomology within otherwise heterologous sequences. The recombination products, in contrast, because they are identified by reconstruction of a functional *supF* gene from two tandem copies of the gene, occur by end joining

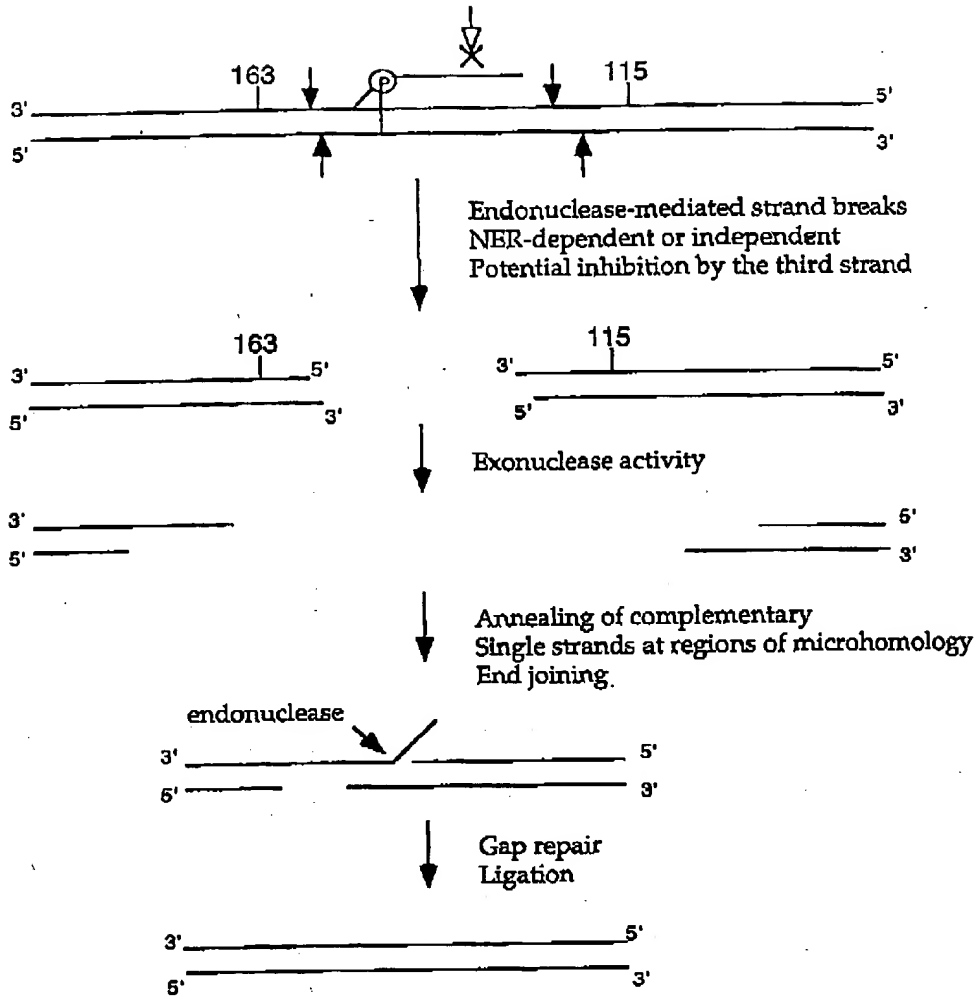



FIG. 7. Model for intramolecular recombination induced by triplex-directed DNA damage. The model illustrates a proposed end-joining pathway in which the third strand-directed psoralen interstrand cross-link is processed into strand breaks, either by NER or by an NER-independent mechanism. (The potential ability of a triple helix to block repair endonuclease activity is also indicated as a complicating factor [61].) The resulting ends are subject to exonuclease digestion, eliminating the mutant sequences and exposing regions of homology capable of joint formation to reconstruct a functional *supF* gene.

within large regions of homology. Nonetheless, it is possible that, in the pSupFAR recombination assay, some of the induced breaks are joined, like the deletions, at microhomologies within heterologous regions, but these products would not be detected in the screen for wild-type *supF* function. (iii) The strand breaks are metabolized to expose single-stranded regions which are capable of strand invasion into homologous sites within another plasmid vector in the cells. The resulting structures could serve as primers for DNA synthesis, promoting further strand displacement and leading to strand exchange, crossing over and recombination. Such a mechanism of break-induced replication was proposed by Li et al. (35) to account for the observation that psoralen cross-links in one plasmid could stimulate repair synthesis in a coincubated undamaged plasmid in human cell extracts. Distinguishing between these pathways will require further studies with defined substrates, with the caveat that the episomal target vector described here may be metabolized by a different set of pathways than would a chromosomal substrate.

Another caveat is that the current work was performed in the presence of SV40 T antigen. T antigen has helicase activity that can unwind triplexes, but such activity was found to require a 3' single-stranded tail (29), which is not present in our triplex substrates. T antigen can also bind and inactivate p53 and so may influence DNA repair (19). However, we have carried out preliminary experiments using a modified shuttle vector in which the T-antigen gene was substituted with a version mutated within the p53 binding domain (30). Essentially similar results were obtained in the recombination assay in both p53 wild-type and mutant human cells (23) (data not shown), suggesting that the p53 status of the cells does not have a major influence on the pso-TFO-induced recombination in this assay. In addition, it should be noted that the SV40 genome is rapidly chromatinized upon transfection into pri-

mate cells (6), so the shuttle vector target approximates the nucleosomal structure of a chromosomal site.

The ability of triple-helix formation, without any other associated DNA damage, to promote recombination reported here provides a novel tool with potential utility for genome modification. Although these experiments were carried out in a model system, with a nonnaturally occurring target sequence, the results should be applicable to any third strand that can bind with high affinity to a genomic site. Recent works demonstrating triplex-mediated targeted mutagenesis of chromosomal loci in mammalian cells support the feasibility of this approach (38, 58). Further, since a number of nucleotide analogs that can significantly enhance triplex formation under physiologic conditions have recently been described (31), this may turn out to be a realistic strategy for genome modification at a variety of sites. More generally, this work raises the possibility that other sequence-specific DNA binding ligands capable of triggering repair may have utility in the genetic manipulation of mammalian cells.

## ACKNOWLEDGMENTS

We thank L. Narayanan, P. Chan, L. Lacroix, K. Vasquez, M. Lin, D. Segal, F. P. Gasparro, L. Cabral, R. Franklin, and S. J. Baserga for their assistance. We also thank K. Kraciner and T. Kunkel for providing cell lines.

This work was supported by the Leukemia Society of America and the NIH (GM54731). A.F.F. was supported in part by the Anna Fuller Foundation.

## REFERENCES

1. Beal, P. A., and P. B. Dervan. 1991. Second structural motif for recognition of DNA by oligonucleotide-directed triple-helix formation. Science 251:1360–1363.
2. Bessho, T., D. Mu, and A. Sancar. 1997. Initiation of DNA interstrand cross-link repair in humans: the nucleotide excision repair system makes dual incisions 5′ to the cross-linked base and removes a 22- to 28-nucleotide-long damage-free strand. Mol. Cell. Biol. 17:6822–6830.
3. Brenneman, M., T. S. Gimble, and J. H. Wilson. 1996. Stimulation of intrachromosomal homologous recombination in human cells by electroporation with site-specific endonucleases. Proc. Natl. Acad. Sci. USA 93:3608–3612.
4. Brookman, K. W., J. E. Lamerdin, M. P. Thelen, M. Hwang, J. T. Reardon, A. Sancar, Z. Q. Zhou, C. A. Walter, C. N. Parris, and L. H. Thompson. 1996. *ERCC4* (*XPF*) encodes a human nucleotide excision repair protein with eukaryotic recombination homologs. Mol. Cell. Biol. 16:6553–6562.
5. Carroll, D., C. W. Lehman, S. Jeong-Yu, P. Dohrmann, R. J. Dawson, and J. K. Trautman. 1994. Distribution of exchanges upon homologous recombination of exogenous DNA in Xenopus laevis oocytes. Genetics 138:445–457.
6. Cereghini, S., and M. Yaniv. 1984. Assembly of transfected DNA into chromatin: structural changes in the origin-promoter-enhancer region upon replication. EMBO J. 3:1243–1253.
7. Chan, P. P., and P. M. Glazer. 1997. Triplex DNA: fundamentals, advances, and potential applications for gene therapy. J. Mol. Med. 75:267–282.
8. Chan, P. P., M. Lin, A. F. Faruqi, J. Powell, M. M. Seidman, and P. M. Glazer. 1999. Targeted correction of an episomal gene in mammalian cells by a short DNA fragment tethered to a triplex-forming oligonucleotide. J. Biol. Chem. 274:11541–11548.
9. Cheng, S., B. Van Houten, H. B. Gamper, A. Sancar, and J. E. Hearst. 1988. Use of psoralen-modified oligonucleotides to trap three-stranded RecA-DNA complexes and repair of these cross-linked complexes by ABC excinuclease. J. Biol. Chem. 263:15110–15117.
10. Chontilas, A., A. Perrin, B. Dujon, and J.-F. Nicolas. 1995. Induction of homologous recombination in mammalian chromosomes by using the I-SceI system of *Saccharomyces cerevisiae*. Mol. Cell. Biol. 15:1968–1973.
11. Cole, R. S. 1973. Repair of DNA containing interstrand crosslinks in Escherichia coli: sequential excision and recombination. Proc. Natl. Acad. Sci. USA 70:1064–1068.
12. Cooney, M., G. Czernuszewicz, E. H. Postel, S. J. Flint, and M. E. Hogan. 1988. Site-specific oligonucleotide binding represses transcription of the human c-myc gene in vitro. Science 241:456–459.
13. Deng, W. P., and J. A. Nickoloff. 1994. Mismatch repair of heteroduplex DNA intermediates of extrachromosomal recombination in mammalian cells. Mol. Cell. Biol. 14:400–406.
14. Donoho, G., M. Jasin, and P. Berg. 1998. Analysis of gene targeting and intrachromosomal homologous recombination stimulated by genomic double-strand breaks in mouse embryonic stem cells. Mol. Cell. Biol. 18:4070–4078.
15. Egholm, M., O. Buchardt, L. Christensen, C. Behrens, S. M. Freier, D. A. Driver, R. H. Berg, S. K. Kim, B. Norden, and P. E. Nielsen. 1993. PNA hybridizes to complementary oligonucleotides obeying the Watson-Crick hydrogen-bonding rules. Nature 365:566–568.
16. Faruqi, A. F., M. Egholm, and P. M. Glazer. 1998. Peptide nucleic acid-targeted mutagenesis of a chromosomal gene in mouse cells. Proc. Natl. Acad. Sci. USA 95:1398–1403.
17. Faruqi, A. F., S. H. Krawczyk, M. D. Matteucci, and P. M. Glazer. 1997. Potassium-resistant triple helix formation and improved intracellular gene targeting by oligodeoxyribonucleotides containing 7-deazaxanthine. Nucleic Acids Res. 25:633–640.
18. Faruqi, A. F., M. M. Seidman, D. J. Segal, D. Carroll, and P. M. Glazer. 1996. Recombination induced by triple helix-targeted DNA damage in mammalian cells. Mol. Cell. Biol. 16:6820–6828.
19. Ford, J. M., and P. C. Hanawalt. 1997. Expression of wild-type p53 is required for efficient global genomic nucleotide excision repair in UV-irradiated human fibroblasts. J. Biol. Chem. 272:28073–28080.
20. Gasparro, F. P., P. A. Havre, G. A. Olack, E. J. Gunther, and P. M. Glazer. 1994. Site-specific targeting of psoralen photoadducts with a triple helix-forming oligonucleotide: characterization of psoralen monoadduct and crosslink formation. Nucleic Acids Res. 22:2845–2852.
21. Hall, J. D., and K. Scherer. 1981. Repair of psoralen-treated DNA by genetic recombination in human cells infected with herpes simplex virus. Cancer Res. 41:5033–5038.
22. Hanson, K. D., and J. M. Sedivy. 1995. Analysis of biological selections for high-efficiency gene targeting. Mol. Cell. Biol. 15:45–51.
23. Havre, P. A., J. Yuan, L. Hedrick, K. R. Cho, and P. M. Glazer. 1995. p53 inactivation by HPV16 E6 results in increased mutagenesis in human cells. Cancer Res. 55:4420–4424.
24. Helene, C. 1991. The anti-gene strategy: control of gene expression by triplex-forming-oligonucleotides. Anti-Cancer Drug Design 6:569–584.
25. Hoy, C. A., E. P. Salazar, and L. H. Thompson. 1984. Rapid detection of DNA-damaging agents using repair-deficient CHO cells. Mutat. Res. 130: 321–332.
26. Ing, N. H., J. M. Beekman, D. J. Kessler, M. Murphy, K. Jayaraman, J. G. Zendegui, M. E. Hogan, B. W. O'Malley, and M. J. Tsai. 1993. In vivo transcription of a progesterone-responsive gene is specifically inhibited by a triplex-forming oligonucleotide. Nucleic Acids Res. 21:2789–2796.
27. Jasin, M. 1996. Genetic manipulation of genomes with rare-cutting endonucleases. Trends Genet. 12:224–228.
28. Koi, M., A. Umar, D. P. Chauhan, S. P. Cherian, J. M. Carethers, T. A. Kunkel, and C. R. Boland. 1994. Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N-methyl-N′-nitro-N-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation. Cancer Res. 54:4308–4312.
29. Kopel, V., A. Pozner, N. Baran, and H. Manor. 1996. Unwinding of the third strand of a DNA triple helix, a novel activity of the SV40 large T-antigen helicase. Nucleic Acids Res. 24:330–335.
30. Kraus, E., L. C. Strong, and M. A. Tainsky. 1995. pZ402, an improved SV40-based shuttle vector containing a T-antigen mutant unable to interact with wild-type p53. Gene 211:229–234.
31. Lacroix, L., J. Lacoste, J. F. Reddoch, J. L. Mergny, D. D. Levy, M. M. Seidman, M. D. Matteucci, and P. M. Glazer. 1999. Triplex formation by oligonucleotides containing 5-(1-propynyl)-2′-deoxyuridine: decreased magnesium dependence and improved intracellular gene targeting. Biochemistry 38:1893–1901.
32. Lehman, C. W., S. Jeong-Yu, J. K. Trautman, and D. Carroll. 1994. Repair of heteroduplex DNA in Xenopus laevis oocytes. Genetics 138:459–470.
33. Lotai, A. G., M. A. Palladino, E. Fromm, V. Rizzo, and J. R. Fresco. 1988. Specificity in formation of triple-stranded nucleic acid helical complexes: studies with agarose-linked polyribonucleotide affinity columns. Biochemistry 27:9108–9112.
34. Levy, D. D., M. Saijo, K. Tanaka, and K. H. Kraemer. 1995. Expression of a transfected DNA repair gene (*XPA*) in xeroderma pigmentosum group A cells restores normal DNA repair and mutagenesis of UV-treated plasmids. Carcinogenesis 16:1557–1563.
35. Li, L., C. A. Peterson, X. Lu, P. Wei, and R. J. Legerski. 1999. Interstrand cross-links induce DNA synthesis in damaged and undamaged plasmids in mammalian cell extracts. Mol. Cell. Biol. 19:5619–5630.
36. Lin, F. L., K. Sperle, and N. Sternberg. 1984. Model for homologous recombination during transfer of DNA into mouse L cells: role for DNA ends in the recombination process. Mol. Cell. Biol. 4:1020–1034.
37. Lin, P. F., E. Bardwell, and P. Howard-Flanders. 1977. Initiation of genetic exchanges in lambda phage-prophage crosses. Proc. Natl. Acad. Sci. USA 74:291–295.
38. Majumdar, A., A. Khorlin, N. Dyatkina, F.-L. M. Lin, J. Powell, J. Liu, Z. Fei, Y. Khripine, K. A. Watanabe, J. George, P. M. Glazer, and M. M. Seidman. 1998. Targeted gene knockout mediated by triple helix forming oligonucleotides. Nat. Genet. 20:212–214.
39. Mansour, S. L., K. R. Thomas, and M. R. Capecchi. 1988. Disruption of the

proto-oncogene int-2 in mouse embryo-derived stem cells: a general strategy for targeting mutations to non-selectable genes. Nature 336:348–352.
40. Maryon, E., and D. Carroll. 1991. Characterization of recombination intermediates from DNA injected into *Xenopus laevis* oocytes: evidence for a nonconservative mechanism of homologous recombination. Mol. Cell. Biol. 11:3278–3287.
41. Moser, H. E., and P. B. Dervan. 1987. Sequence-specific cleavage of double helical DNA by triple helix formation. Science 238:645–650.
42. Parris, C. N., and M. M. Seidman. 1992. A signature element distinguishes sibling and independent mutations in a shuttle vector plasmid. Gene 117:1–5.
43. Praseuth, D., L. Perrouault, T. Le Doan, M. Chassignol, N. Thuong, and C. Helene. 1988. Sequence-specific binding and photocrosslinking of alpha and beta oligodeoxynucleotides to the major groove of DNA via triple-helix formation. Proc. Natl. Acad. Sci. USA 85:1349–1353.
44. Puchta, H., B. Dujon, and B. Hohn. 1996. Two different but related mechanisms are used in plants for the repair of genomic double-strand breaks by homologous recombination. Proc. Natl. Acad. Sci. USA 93:5055–5060.
45. Reardon, J. T., P. Spielmann, J. C. Huang, S. Sastry, A. Sancar, and J. E. Hearst. 1991. Removal of psoralen monoadducts and crosslinks by human cell free extracts. Nucleic Acids Res. 19:4623–4629.
46. Roth, D. B., and J. H. Wilson. 1986. Nonhomologous recombination in mammalian cells: role for short sequence homologies in the joining reaction. Mol. Cell. Biol. 6:4295–4304.
47. Rouet, P., F. Smih, and M. Jasin. 1994. Expression of a site-specific endonuclease stimulates homologous recombination in mammalian cells. Proc. Natl. Acad. Sci. USA 91:6064–6068.
48. Saffran, W. A., C. R. Cantor, E. D. Smith, and M. Magdi. 1992. Psoralen damage-induced plasmid recombination in Saccharomyces cerevisiae: dependence on RAD1 and RAD52. Mutat. Res. 274:1–9.
49. Sancar, A. 1996. DNA excision repair. Annu. Rev. Biochem. 65:43–81.
50. Sandor, Z., and A. Bredberg. 1995. Triple helix directed psoralen adducts induce a low frequency of recombination in an SV-40 shuttle vector. Biochim. Biophys. Acta 1263:235–240.
51. Segal, D. J., and D. Carroll. 1995. Endonuclease-induced, targeted homologous extrachromosomal recombination in Xenopus oocytes. Proc. Natl. Acad. Sci. USA 92:806–810.
52. Segal, D. J., A. F. Faruqi, P. M. Glazer, and D. Carroll. 1997. Processing of targeted psoralen cross-links in *Xenopus* oocytes. Mol. Cell. Biol. 17:6645–6652.
53. Sladek, F. M., M. M. Munn, W. D. Rupp, and P. Howard-Flanders. 1989. In vitro repair of psoralen-DNA cross-links by RecA, UvrABC, and the 5′-exonuclease of DNA polymerase I. J. Biol. Chem. 264:6755–6765.
54. Tsujimura, T., V. M. Maher, A. R. Godwin, R. M. Liskay, and J. J. McCormick. 1990. Frequency of intrachromosomal homologous recombination induced by UV radiation in normally repairing and excision repair-deficient human cells. Proc. Natl. Acad. Sci. USA 87:1566–1570.
55. Umar, A., J. C. Boyer, D. C. Thomas, D. C. Nguyen, J. I. Risinger, J. Boyd, Y. Ionov, M. Perucho, and T. A. Kunkel. 1994. Defective mismatch repair in extracts of colorectal and endometrial cancer cell lines exhibiting microsatellite instability. J. Biol. Chem. 269:14367–14370.
56. Van Houten, B., H. Gamper, J. E. Hearst, and A. Sancar. 1988. Analysis of sequential steps of nucleotide excision repair in Escherichia coli using synthetic substrates containing single psoralen adducts. J. Biol. Chem. 263:16553–16560.
57. Van Houten, B., H. Gamper, S. R. Holbrook, J. E. Hearst, and A. Sancar. 1986. Action mechanism of ABC excision nuclease on a DNA substrate containing a psoralen crosslink at a defined position. Proc. Natl. Acad. Sci. USA 83:8077–8081.
58. Vasquez, K. M., G. Wang, P. A. Havre, and P. M. Glazer. 1999. Chromosomal mutations induced by triplex-forming oligonucleotides in mammalian cells. Nucleic Acids Res. 27:1176–1181.
59. Vasquez, K. M., and J. H. Wilson. 1998. Triplex-directed modification of genes and gene activity. Trends Biochem. Sci. 23:4–9.
60. Vos, J. M., and P. C. Hanawalt. 1989. DNA interstrand cross-links promote chromosomal integration of a selected gene in human cells. Mol. Cell. Biol. 9:2897–2905.
61. Wang, G., and P. M. Glazer. 1995. Altered repair of targeted psoralen photoadducts in the context of an oligonucleotide-mediated triple helix. J. Biol. Chem. 270:22595–22601.
62. Wang, G., D. D. Levy, M. M. Seidman, and P. M. Glazer. 1995. Targeted mutagenesis in mammalian cells mediated by intracellular triple-helix formation. Mol. Cell. Biol. 15:1759–1768.
63. Wang, G., M. M. Seidman, and P. M. Glazer. 1996. Mutagenesis in mammalian cells induced by triple helix formation and transcription-coupled repair. Science 271:802–805.
64. Wang, Y. Y., V. M. Maher, R. M. Liskay, and J. J. McCormick. 1988. Carcinogens can induce homologous recombination between duplicated chromosomal sequences in mouse L cells. Mol. Cell. Biol. 8:196–202.
65. White, S., J. W. Szewczyk, J. M. Turner, E. E. Baird, and P. B. Dervan. 1998. Recognition of the four Watson-Crick base pairs in the DNA minor groove by synthetic ligands. Nature 391:468–471.